DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
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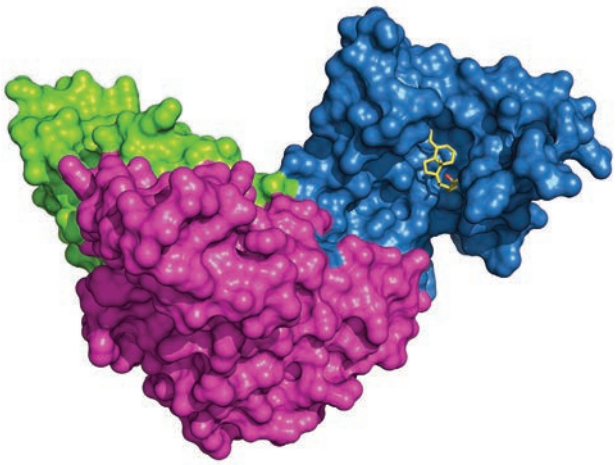
ELISA SAARNIO

ASSOCIATIONS AMONG VITAMIN D BINDING PROTEIN GENE POLYMORPHISMS, TOTAL, FREE AND BIOAVAILABLE 25-HYDROXYVITAMIN D AND SKELETAL OUTCOMES – STUDIES IN CHILDREN, ADOLESCENTS, AND MIDDLE-AGED FINNS



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Associations among Vitamin D Binding Protein Gene Polymorphisms, Total, Free and Bioavailable 25-Hydroxyvitamin D and Skeletal Outcomes – Studies in Children, Adolescents, and Middle-aged Finns

Elisa Saarnio

ACADEMIC DISSERTATION

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Supervisors

Professor Christel Lamberg-Allardt, PhD
Department of Food and Nutrition
University of Helsinki

Senior Researcher Minna Pekkinen, PhD
Folkhälsan Research Center, Helsinki, Finland
Children's Hospital and Research Program for Clinical and Molecular Metabolism,
Faculty of Medicine, University of Helsinki

Reviewers

Professor, Harri Niinikoski, MD
Departments of Physiology and Pediatrics
University of Turku

Associate Professor, Jyrki Virtanen, PhD
Institute of Public Health and Clinical Nutrition
University of Eastern Finland

Opponent

Professor, Marjukka Kolehmainen, FT
Institute of Public Health and Clinical Nutrition
University of Eastern Finland

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To my family

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ORIGINAL PUBLICATIONS

TIIVISTELMÄ, Finnish Summary

D-vitamiini on elimistölle välttämätön vitamiini. D-vitamiinia muodostuu iholla auringon ultraviolettisäteilyn avulla sekä sitä saadaan ravinnosta. D-vitamiini muuttuu maksassa 25-hydroksi- D-vitamiiniksi (25(OH)D), joka on pääasiallinen veressä kiertävä muoto ja sen jälkeen munuaisissa aktiiviseksi 1,25-hydroksi D-vitamiiniksi (1,25(OH)D), joka toimii kalsium- ja fosfaattiaineenvaihdunnan säätelijänä. D-vitamiinin kuljettajaproteiini (DBP) kuljettaa D-vitamiinimuotoja verenkierrossa ja kohdekudoksiin. Pieni määrä D-vitamiinia on myös vapaana verenkierrossa. Vapaan D-vitamiinin on ehdotettu olevan pienestä pitoisuudestaan huolimatta aktiivisempi muoto, koska se pääsee soluihin ilman reseptoria. DBP:n kyky sitoa 25(OH)D:ta vaihtelee DBP:n genotyypin mukaan ja voi vaikuttaa siten proteiiniin sitoutuneen ja vapaan 25(OH)D:n pitoisuuksiin veressä. Tutkimuksissa on havaittu, että lihavilla on alhaisemmat seerumin D-vitamiinipitoisuudet kuin normaalipainoisilla. D-vitamiinilla on tärkeä rooli luun kasvussa ja luuston ylläpidossa koko ihmisen elinkaaren. D-vitamiinin puutos lapsuudessa ja nuoruudessa voi vaikuttaa luuston kehitykseen ja kasvuun. Aikuisuudessa D-vitamiinin puutos voi olla osallisena luukatoon. DBP:n deglykosyloituneella muodolla on raportoitu olevan rooli luun muodostumisessa.

Tämän väitöskirjan tavoite oli tutkia DBP:n geneettisen vaihtelun vaikutusta D-vitamiiniaineenvaihduntaan ja luustoon. Lisäksi tavoite oli tutkia eroaako lihavien D-vitamiiniaineenvaihdunta normaalipainoisista ja onko mahdollisilla eroilla vaikutuksia luustoon.

Tutkimusaineisto koostuu 233 suomalaisesta lapsesta ja nuoresta (7–19 vuotiaita) (Osatyö I), sekä 622 suomalaisesta naisesta ja miehestä (37–47 vuotiaita) (Osatyöt II ja III). Osatyössä III naiset ja miehet jaettiin painoindeksin mukaan normaalipainoisiin, ylipainoisiin ja lihaviin. Kaikissa tutkimuksissa kerättiin D-vitamiinin ja kalsiumin saantitiedot sekä tietoa lääkityksistä sekä fyysisestä aktiivisuudesta. Paastoverinäytteestä määritettiin 25(OH)D, lisäkilpirauhashormoni (PTH), albumiini sekä luun aineenvaihdunnan merkkiaineet. Lisäksi määritettiin DBP:n pitoisuudet (osatöissä II - III) sekä sen geenipolymorfioita (osatyöt I ja II). Vapaan 25(OH)D:n pitoisuudet määritettiin laskennallisesti (osatöissä II ja III) ja lisäksi tutkimuksessa II 25(OH)D pitoisuudet vakioitiin genotyyppikohtaisilla affiniteettivakioilla. Luun mineraalitiheys mitattiin kaksienergiaisella röntgenabsorptiometrialla (DXA) (osatyö I) ja perifeerisellä kvantitatiivisella kerroskuvausmenetelmällä (pQCT) (osatyöt I ja III).

Lapsilla ja nuorilla (Osatyö I) DBP:n genotyypillä *GC* 2/2 oli matalimmat 25(OH)D ja PTH-pitoisuudet. Pojilla DBP:n genotyyppi oli negatiivisesti yhteydessä lonkan luun mineraalipitoisuuteen ja lannerangan luun mineraalitiheyteen. Molemmilla sukupuolilla DBP:n genotyyppi oli yhteydessä lannerangan luun mineraalipitoisuuteen ja luun vahvuusindeksiin. Aikuisilla (Osatyö II) DBP:n sekä kokonais- ja vapaan 25(OH)D:n sekä PTH:n pitoisuudet erosivat merkitsevästi DBP:n genotyyppien välillä. DBP:n pitoisuus oli alhaisin genotyypillä *GC*2/2. Matalimmat 25(OH)D pitoisuudet havaittiin diplotyypeillä *GC*1S/2, *GC*1S/1F ja *GC* 2/2. Käytettäessä genotyyppiadjustoituja arvoja, genotyypillä *GC*2/2 havaittiin korkeimmat vapaan 25(OH)D:n pitoisuudet. Yllättäen PTH pitoisuus oli myös matalin genotyypillä *GC*2/2. Osatyössä III lihavilla naisilla oli matalammat vapaan 25(OH)D:n pitoisuudet kuin normaalipainoisilla. Miehillä sekä sitoutuneen, että vapaan 25(OH)D:n pitoisuudet olivat matalammat lihavilla kuin normaalipainoisilla. DBP- ja PTH-pitoisuudet olivat korkeammat lihavilla naisilla verrattuna normaalipainoisiin naisiin. Yhdistettäessä naisten ja miesten ryhmä, lihavien ryhmällä oli myös korkeammat DBP- ja PTH-pitoisuudet kuin normaalipainoisilla. Lihavilla naisilla 25(OH)D oli negatiivisesti yhteydessä hohkaluun tiheyteen värttinäluussa ja kuoriluun lujuusindeksiin sääriluussa. Lisäksi vapaan 25(OH)D:n pitoisuudet olivat lihavilla käänteisessä yhteydessä värttinäluun distaalipään lujuusindeksiin, värttinäluun proksimaalipään tiheyteen kuoriluussa sekä sääriluun proksimaalipään tiheyteen kuoriluussa.

Saadut tulokset osoittavat, että DBP:n geneettinen vaihtelu vaikuttaa sekä vapaan, että sitoutuneen seerumin 25(OH)D:n pitoisuuksiin sekä DBP:n ja PTH:n pitoisuuksiin. Havainnolla voi olla merkitystä, kun D-vitamiinitilaa sekä sen metaboliaa ja toimintoja tutkitaan. DBP:n geneettinen vaihtelu voi olla yksi tekijä, joka vaikuttaa luumassan kertymiseen nuoruudessa. Lihavuuden yhteys sitoutuneen- ja vapaan 25(OH)D:n, DBP:n ja PTH:n pitoisuuksiin viittaavat siihen, että lihavien D-vitamiiniaineenvaihdunta eroaa normaalipainoisten aineenvaihdunnasta. Muutokset D-vitamiinin aineenvaihdunnassa voivat vaikuttaa negatiivisesti luustoon.

ABSTRACT

Vitamin D is a crucial vitamin for the human body. Vitamin D can be derived from ultraviolet light -induced synthesis in the skin and from the diet. Vitamin D is metabolized in the liver to the main circulating form, 25-hydroxyvitamin D (25(OH)D), and then in the kidney to 1,25-dihydroxyvitamin D (1,25(OH)D), which is the endocrine regulator of calcium and phosphate homeostasis. Vitamin D binding protein (DBP) transports vitamin D in the circulation and into target tissues. A small amount of vitamin D is free in the circulation. Hence, these free metabolites have been suggested to be more active because they can enter the cells without a receptor. Genetic variation of DBP coding gene affects the protein's ability to bind 25(OH)D, thereby influencing DBP bound and free 25(OH)D concentrations in the circulation. Several studies have reported that vitamin D concentrations are lower in obese individuals than in their normal-weight peers. Vitamin has a significant role in bone growth and for maintenance of the skeleton throughout life. Vitamin D deficiency in childhood and adolescence affects bone development and growth. In adulthood, vitamin D deficiency can contribute to bone loss. The deglycosylated form of DBP has been reported to have a role in bone formation.

The purpose of this thesis was to investigate genetic variation of DBP on vitamin D metabolism and bone. Other goals were to study whether obese individuals differ in their vitamin D metabolism from the normal-weight individuals and do these potential differences affect bone.

The study population consisted of 233 Finnish children and adolescents (7-19 years) (Study I) and 622 Finnish women and men (37–47 years) (Studies II and III). In Study III, the adults were stratified by body mass index into normal-weight, overweight, and obese.

The data on dietary intake of vitamin D and calcium, medications and physical activity were collected. 25(OH)D, PTH, albumin, and bone turnover markers were determined from fasting blood samples. In addition, DBP concentration (Study II- III) and DBP gene polymorphisms (Study I and II) were determined. Free 25(OH)D concentrations were calculated (Study II - III) and in Study II the values were adjusted with genotype specific affinity values. Bone mineral density was measured with Dual-Energy X-ray-absorptiometry (DXA) (Study I) and peripheral quantitative computed tomography (pQCT) in (Study I-III).

In children and adolescents (Study I), 25(OH)D and PTH concentrations were lowest in genotype *GC*2/2. In boys, DBP genotype was negatively associated with hip bone mineral content and lumbar spine bone mineral density. In both girls and boys, DBP genotype was associated with lumbar spine bone mineral content and strength strain index. In adults (Study II), significant variation were found among DBP gene variants among DBP, total, free, and bioavailable 25(OH)D, and PTH. DBP concentration was lowest in genotype *GC*2/2. 25(OH)D concentrations were lowest in *GC* diplotypes 1S/2, 1S/1F, and 2/2, but free and bioavailable 25(OH)D concentrations were highest in *GC*2/2 when a genotype-specific binding constant was used. Unexpectedly, the same gene variant was also associated with low PTH concentrations.

In Study III, free and bioavailable 25(OH)D concentrations were lower in obese women than in normal-weight women. In men, both bound and free 25(OH)D were lower in obese individuals than in those of normal-weight. In addition, DBP and PTH were higher in obese women and when men and women were combined in the same group. In obese women, 25(OH)D was negatively associated with distal radius trabecular density and tibial shaft cortical strength index (CSI). Furthermore, free and bioavailable 25(OH)D were negative determinants of distal radius CSI, radial shaft cortical density, and tibial shaft CSI. Bioavailable 25(OH)D negatively associated with distal radius CSI, radial shaft cortical density, and tibial shaft CSI among obese subjects.

In conclusion, genetic variation in DBP coding gene is associated with DBP bound and unbound 25(OH)D, DBP and PTH concentrations. This finding may be relevant, when vitamin D status, metabolism, and actions are investigated. In addition, the results show also that genetic variation of DBP can be one factor affecting bone mass accrual in adolescence.

Associations of body mass index with total, free and bioavailable 25(OH)D, DBP, and PTH concentrations demonstrate a difference in vitamin D metabolism between obese and normal-weight individuals. Furthermore, altered vitamin D metabolism may have a negative influence on bone.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-III):

I Pekkinen M*, **Saarnio E***, Viljakainen HT, Kokkonen E, Jakobsen J, Cashman K, Mäkitie O, Lamberg-Allardt C. Vitamin D binding protein genotype is associated with serum 25-hydroxyvitamin D and PTH concentrations, as well as bone health in children and adolescents in Finland. PLoS One. 2014; 30;9(1). Doi: 10.1371/journal.pone.0087292.

* equal contribution for first authorship

II **Saarnio E**, Pekkinen M, Itkonen ST, Kemi V, Karp H, Kärkkäinen M, Mäkitie O, Lamberg-Allardt C. Serum parathyroid hormone is related to genetic variation in vitamin D binding protein with respect to total, free, and bioavailable 25-hydroxyvitamin D in middle-aged Caucasians – a cross-sectional study. BMC Nutrition 2016; 2:46. Doi 10.1186/s40795-016-0085-3.

III **Saarnio E**, Pekkinen M, Itkonen ST, Kemi V, Karp H, Ivaska K, Risteli J, Koivula MK, Kärkkäinen M, Mäkitie O, Sievänen H, Lamberg-Allardt C. Low free 25-hydroxyvitamin D and high vitamin D binding protein and parathyroid hormone in obese Caucasians. A complex association with bone? PLoS One. 2018; 28;13(2):e0192596. Doi: 10.1371/journal.pone.0192596.

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Contribution of the authors to Studies I-III

I: HTV MP OM CLA conceived and designed the experiments, HTV ES MP JJ KC OM and CLA performed the experiments, MP ES and EK analyzed the data, ES wrote the paper together with MP and CLA. ES, MP, and CLA are responsible for the integrity of data analysis. All authors read, reviewed and approved the final manuscript.

II: ES, MP, STI, VK, HK, MK, OM, CLA designed the study, ES, MP, STI, VK, HK, MK, CLA conducted the study. ES, MP, STI, VK, HK, MK, CLA collected the data. ES and MP analyzed the data. ES, MP, and CLA interpreted the data. ES drafted the manuscript with the help of MP and CLA. ES, MP, and CLA are responsible for the integrity of data analysis. All authors read, reviewed and approved the final manuscript.

III: ES, MP, STI, VK, HK, MK, CLA designed the study. ES, MP, STI, VK, HK, MK, MKK, KKI, JR, CLA collected the data. ES analyzed the data. ES, CLA and MP interpreted the data. ES drafted the manuscript with the help of MP and CLA. ES, MP, and CLA are responsible for the integrity of data analysis. All authors read, reviewed and approved the final manuscript.

ABBREVIATIONS

ALP, alkaline phosphatase

ANCOVA, analysis of covariance

ANOVA, analysis of variance

BALP, bone specific alkaline phosphatase

BMC, bone mineral content

BMD, bone mineral density

BMI, body mass index

BSI, bone strength index

Ca, calcium

C5a, complement component 5a

CLIA, chemiluminescence assays

CorD, cortical density

CPBA, competitive protein-binding assays

CTX-I, carboxy-terminal crosslinked telopeptide of type I collagen

CSI, cortical strength index

CYP27B1, 25-hydroxyvitamin D 1-alpha-hydroxylase, cytochrome p450 27B1

CYP2R1, vitamin D 25-hydroxylase, cytochrome P450 2R1

DEQAS, vitamin D external quality assessment scheme

DBP, vitamin D binding protein

DHCR7, 7-dehydrocholesterol reductase

DXA, Dual Energy X-ray Absorptiometry

ELISA, Enzyme Linked Immunosorbent assay

FFQ, Food Frequency Questionnaire

FGF23, fibroblast growth factor 23

GC gene, vitamin D binding protein coding gene

GC-globulin, group-specific component, vitamin D binding protein

GC-MAF, GC protein-derived macrophage activating factor

GWAS, Genome-Wide Association Study

1,25(OH)D, 1,25-hydroxy-vitamin D_3, calcitriol

25(OH)D, 25-hydroxy-vitamin D, calcidiol

HPLC, high-performance liquid chromatography

iPTH, intact parathyroid hormone

IOM, Institute Of Medicine of United States of America

LC-MS/MS, liquid chromatography–tandem mass spectrometry

NTX-I, amino-terminal crosslinked telopeptide of type I collagen

OPG, osteoprotegerin

PICP, procollagen type I c-terminal propeptide

PINP, procollagen type I N-terminal propeptide

PTH, parathyroid hormone

pQCT, peripheral Quantitative Computed Tomography

RANKL, receptor activator of nuclear factor kappa-B ligand

RIA, radioimmunoassay

SISU, Sequencing Initiative Suomi

SNP, single nucleotide polymorphism

SSI, strength strain index

TraD, trabecular density

UVB, ultraviolet B

VDSP, Vitamin D Standardization Program

1 INTRODUCTION

Vitamin D has received attention in many research areas in recent years. Vitamin D deficiency is common worldwide and has been suggested to be associated with such diseases as cancer, autoimmune disorders, hypertension, metabolic syndrome, and diabetes (Holick *et al.* 2008, Cashman *et al.* 2016). Vitamin D status is influenced by several factors such as sunshine exposure, diet, latitude, and genetic factors (Holick 2008, Wang 2010).

In Finland, vitamin D deficiency has earlier been common both due to low dietary vitamin D intake (Lamberg-Allardt *et al.* 2001, Männistö *et al.* 2002) and to limited ultraviolet B-radiation (UVB) radiation during winter needed for dermal synthesis of vitamin D. Inadequate vitamin D status (25(OH)D < 40 nmol/L) was recognized to be a potential public health problem among healthy adults in Finland (Lamberg-Allardt *et al.* 2001). Vitamin D status of the Finnish adult population has improved considerably from 2000 to 2011, mostly due to fortification of fluid milk products and increased use of vitamin D supplements (Jääskeläinen *et al.* 2017).

Vitamin D is a fat-soluble vitamin produced in the skin by UVB-radiation or obtained from certain foods and supplements. The circulating form of vitamin D, 25(OH)D is produced in the liver and is converted to the active form known as 1,25-dihydroxyvitamin D (1,25(OH)D) mostly in the kidney. 1,25(OH)D can also be made in several other extrarenal sites. Vitamin D binding protein (DBP) transports vitamin D in the circulation. Only a small proportion of vitamin D is free in the circulation (Bikle *et al.* 1986). The megalin/cubilin receptors at the cell surface take up 25(OH)D/DBP complex into renal proximal cells. 25(OH)D has commonly been considered the best biomarker of vitamin D status. However, studies have suggested that unbound, free 25(OH)D could be the more active form of vitamin D (Al Oanzi *et al.* 2006, Chun *et al.* 2014, Johnsen *et al.* 2014, Powe *et al.* 2011).

Vitamin D, i.e. 1,25(OH)D has important roles in calcium homeostasis. The most important one is the regulation of intestinal calcium absorption - 1,25(OH)D increases it when more calcium is needed in the body. Other mechanism in which 1,25(OH)D is involved in a hypercalcemic situation includes calcium reabsorption in nephrons and stimulation of osteoblasts to activate osteoclasts to resorb bone to raise serum calcium concentration. Vitamin D also suppresses parathyroid hormone (PTH) production, which also has important roles of its own in calcium homeostasis, by affecting both calcium

renal reabsorption and bone resorption. Variation in the DBP coding gene can affect vitamin D status (Arnaud & Constans 1993). In vitamin D deficiency, low intestinal calcium absorption leads to lower serum calcium concentrations followed by higher PTH concentrations, which increases bone resorption. This has been suggested to enhance the development of osteoporosis.

Vitamin D is an important factor for bone health and preventing osteoporosis. In women bone loss increases especially around menopause due to decreasing oestrogen concentrations. In men the bone loss is steadier. DBP has been associated with bone mineral density (BMD) through the genetic polymorphism that can influence the binding and transport of 25(OH)D and other metabolites in the circulation. It has also been suggested that DBP could work directly as a precursor protein for macrophage activating factor (GC-MAF), which activates osteoclasts (Yamamoto 1993, Schneider 1995).

Childhood and adolescence are perhaps the most important phases for bone development. Low peak bone mass can negatively affect bone remodelling and increase the risk of having osteoporosis in older age.

Obesity is a worldwide problem and it is often associated with many other diseases such as metabolic syndrome and insulin resistance (WHO 2000). Obesity has also been associated with vitamin D deficiency most likely due to the sequestration of vitamin D in fat tissue (Vanlint 2013).

This study aims to investigate the genetic polymorphisms of DBP and vitamin D metabolism in Finnish children and adolescents as well as in Finnish middle-aged subjects and the associations of vitamin D and genetic polymorphisms of DBP with bone health. The first two studies investigate the DBP gene polymorphism and calcium homeostasis and bone health. The third study focuses on vitamin D metabolism in obese middle-aged men and women and the effect of free vitamin D metabolites on bone health. The principal focus of these studies is on investigating the several roles of vitamin D binding protein in vitamin D status and bone parameters. A future goal is detecting individuals who are at higher risk of developing vitamin D deficiency.

2 REVIEW OF THE LITERATURE

2.1 Vitamin D

2.1.1 Sources and current and past recommendations

Vitamin D_3 (cholecalciferol) is produced from 7-dehydrocholesterol in the skin during sunlight i.e. UVB exposure which is the physiological route for vitamin D in humans. However, food derived vitamin D is important, when sunlight is not always available. Major natural dietary sources of vitamin D_3 are of animal origin, such as many species of fish, animal and fish liver and inner organs, and egg yolk. Some plant-based dietary sources, containing vitamin D_2 (ergocalciferol), such as wild mushrooms, can be produced in baker´s yeast and cultivated mushrooms upon UVB irradiation. Fortified foods are important dietary sources in many countries, for instance, milk and fat spreads are fortified with either vitamin D_3 or vitamin D_2. The ministry of Trade and Industry in Finland recommended by decree voluntary fortification of some specific food stuffs in 2003: fluid milk products (i.e. milk, sour milk, yoghurt and their non-milk equivalents) could be fortified with 0.5 µg vitamin D_3/100 ml and all fat spreads with 10 µg/100 g. In 2010, the recommendation for the fortification increased to 1 µg vitamin D_3/100 ml and 20µg/100g for fluid milk products and spreads, respectively.

The daily recommendation for vitamin D intake from food and supplements set by the Finnish National Nutrition Council was 7.5 µg for persons aged 3-60 years between 2004 and 2013 and from 2014 on 10 µg for children and adolescents (2-18 years) and 10 µg for adults (18-74 years) based on the recommendations of the Nordic Council of Ministries from 2004 and 2012, respectively (Nordic Council of Ministries 2004, National Nutrition Council 2005, National Nutrition Council 2014, Nordic Council of Ministers 2013).

The Institute of Medicine of USA (IOM) 2011 thresholds for serum 25(OH)D concentrations are ≤ 30 nmol/L=vitamin D deficiency, 30–49.9 nmol/L=insufficiency, and ≥50 nmol/L=sufficiency (IOM 2011). Many clinicians agree that clinical vitamin D

deficiency only occurs when serum 25(OH)D is lower than 25 or 30 nmol/L (Lips 2001, Need *et al.* 2008, EFSA 2016). There are several different views on optimal serum 25(OH)D concentrations for skeletal effects and muscle strength and non-classical effects. According to the IOM, the concentration should be at least 50 nmol/L (EFSA 2016) but according to the Endocrine Society at least 75 nmol/L (Holick *et al*. 2011). According to Jääskeläinen and colleagues, vitamin D status of the Finnish adult population has improved significantly from 2000 to 2011 based on the Health 2000 and 2011 studies (Jääskeläinen *et al*. 2017). Based on their study, 91 % of supplement nonusers in Health 2011 study, who consumed fluid milk products, fat spreads, and fish based on Finnish nutrition recommendations reached vitamin D status that is considered sufficient (25(OH)D ≥ 50 nmol/L). Food fortification, especially of fluid milk products, and increased use of vitamin D supplements are likely reasons for the improvement in vitamin D status.

2.1.2 Vitamin D metabolism

Vitamin D is a common name for a group of substances, the most important of which are cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2). Vitamin D belongs to a subclass of seco-steroids, with a structure similar to that of steroids. UVB radiation converts 7-dehydrocholesterol (provitamin D) to previtamin D_3, which is isomerized into vitamin D_3. Clothing and sun-screen as well as melanin in the skin blocks UVB radiation and limits dermal vitamin D production. There is a difference in the intensity of UVB radiation depending on the season and latitude. Vitamin D_3 synthesized in the skin is transported in blood bound to vitamin D binding protein. Ingested vitamin D_2 and D_3 are absorbed primarily in the ileum and transported in chylomicrons and lipoproteins via lymphatic vessels with other fats, and emptied into the thoracic duct (Vieth 1999). Vitamin D metabolism is described in Figure 1.

25-hydroxyvitamin D

Vitamin D is hydroxylated with vitamin D 25-hydroxylase (CYP2R1) into 25(OH)D in the liver and other tissues (Bikle 2014). In the circulation, DBP binds up to 90 % of the 25(OH)D (Bikle *et al*. 1986, Nykjaer *et al*. 1999). Serum 25(OH)D concentration is considered the best marker for vitamin D status of the body. It has three structurally different forms: $25(OH)D_3$, $25(OH)D_2$, and 3 epi-$25(OH)D_2$/3 epi-$25(OH)D_3$. Megalin is a transmembrane protein that functions as a cell-surface receptor for the DBP-25(OH)D complex. When the DBP-25(OH)D complex binds to megalin, 25(OH)D is released and enters the proximal tubular cells of the kidneys and some other cells through endocytosis (Nykjaer *et al*. 1999). 25(OH)D is transformed into $1,25(OH)_2D_3$ or $1,25(OH)_2D_2$ mainly in the kidney, but also in other tissues. Serum 25(OH)D has much longer half-life (1-2 months) than 1,25(OH)D (4 hours) and is therefore relatively stable. The concentration of serum 25(OH)D is almost 1000-fold of that of 1,25(OH)D (Lips 2007). Serum concentrations of 25(OH)D correlate better with a number of clinical outcomes (e.g. cancer rates, immune function, cardiovascular health, and mortality) than serum 1,25(OH)D concentrations (Holick *et al*. 2008).

1,25-hydroxyvitamin D

25(OH)D is converted into an active vitamin D metabolite, 1,25(OH)D, in the kidney and various other cells e.g. epithelial cells of the skin, lungs, breast, intestine, and prostate; endocrine cells of the parathyroid gland, pancreatic islets, thyroid, testes, ovary, and placenta; macrophages; T and B lymphocytes and dendritic cells and bone cells, including osteoblasts and chondrocytes (Bikle *et al*. 2014). Compared to 25(OH)D, 1,25(OH)D has a very short lifespan. It is an important regulator of calcium homeostasis and is responsible for the mineral balance of the skeleton. The production of 1,25(OH)D is regulated by a negative feedback system, where PTH stimulates and calcium, phosphate, and fibroblast growth factor 23 (FGF23) inhibit its production. When the concentration of calcium in the circulation falls, PTH is released by the parathyroid gland. PTH stimulates 25-hydroxyvitamin D 1-alpha-hydroxylase (CYP27B1) to make more 1,25(OH)D from 25(OH)D. Thus, elevated calcium suppresses CYP27B1 via suppression of PTH, and elevated phosphate suppresses CYP27B1 by stimulating FGF23. 1,25(OH)D binds to vitamin D receptor (VDR) inside cells and induces the

absorption of calcium and phosphate in the epithelium of the small intestine. The increase of 1,25(OH)D causes an increase in calcium transport within the intestine, bone and kidney and thus, plasma calcium levels rise. The calcium sensing receptor of the parathyroid gland senses the increased levels of calcium in plasma and the secretion of PTH is suppressed. 1,25(OH)D can also directly suppress the secretion of PTH (Bikle 2012).

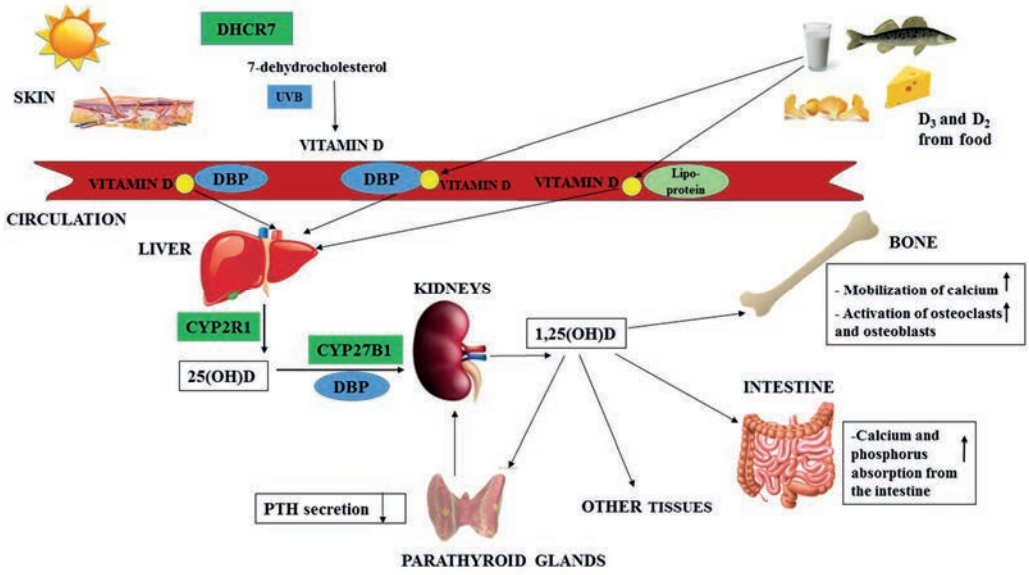

Figure 1 Vitamin D metabolism and actions. Vitamin D is ingested in the diet or produced in the skin with exposure to UVB radiation. DHCR7 converts 7-dehydrocholesterol into vitamin D. Vitamin D (D_2 or D_3) is converted by *CYP2R1* to 25(OH)D in the liver. 25(OH)D is converted by CYP27B1 to 1,25(OH)D in the kidneys and several other cells. 1,25(OH)D increases intestinal calcium and phosphorus absorption and stimulates osteoblast and osteoclast production in bone. In the parathyroid glands, 1,25(OH)D decreases PTH synthesis and release. Modified from (Holick 2006).

Assessment of 25(OH)D

A hydrophobic nature, three structurally different forms, and tight binding to DBP render measurement of 25(OH)D challenging (Binkley & Carter 2017). There are several methods to determine 25(OH)D in serum such as high-performance liquid chromatography with UV detection (HPLC/UV), liquid chromatography–tandem mass spectrometry (LC-MS/MS), and immunoassays (radioimmunoassay (RIA), competitive protein-binding assays (CPBA), enzyme-linked immunosorbent assays (ELISA), chemiluminescence assays (CLIA). Liquid chromatography, followed by LC-MS/MS is more precise than immunoassays and has therefore become the gold standard for the measurement of vitamin D. With these two methods, it is possible to measure 25(OH)D_3 and 25(OH)D_2 separately. They can also detect other vitamin D metabolites such as 24,25-dihydroxyvitamin D (24,25(OH) D). LC-MS/MS analyses have revealed that immunoassays are marred by interference of 24,25$(OH)_2$D, particularly at higher 25(OH)D concentrations. This leads to excessive 25(OH)D concentrations in the analysis (Binkley & Carter 2017). Furthermore, the 3-epi-25(OH)D metabolite may interfere in some immunoassays (Bailey *et al.* 2014, Binkley & Carter 2017, Calvo&Lamberg-Allardt 2017).

The Vitamin D External Quality Assessment Scheme (DEQAS) was founded to guarantee the analytical reliability of 25(OH)D and 1,25(OH)D assays. DEQAS has revealed substantial differences among different vitamin D measurement methods both within and between laboratories. DEQAS has distributed serum to around 1200 laboratories. Participating laboratories receive the results as the difference between their laboratory and the overall mean value (http://www.deqas.org/, Carter 2010). The Vitamin D Standardization Program (VDSP) was established in 2010 by the National Institutes of Health Office of Dietary Supplements in USA. VDSP is an international collaboration and its purpose is to standardize the measurements of vitamin D status among different laboratories. The aim is to have comparable laboratory measurements of vitamin D over time, location, and laboratory procedure (Binkley *et al.* 2014, Cashman *et al.* 2015).

2.1.3 Genes related to vitamin D metabolism

Genome-Wide Association Studies (GWAS) and candidate gene studies have identified several genes involved in vitamin D synthesis, metabolism and transport (Shea *et al.* 2009, Ahn *et al*. 2010, Wang *et al.* 2010). Genetic variation in GC (vitamin D binding protein), CYP2R1 (Vitamin D 25-hydroxylase), 7-dehydrocholesterol reductase (DHCR7), CYP24A1 (1,25-dihydroxyvitamin D 24-hydroxylase), 1α-hydroxylase enzyme (CYP27B1, and VDR coding genes has been associated with vitamin D status (Engelman *et al*. 2008, Wang *et al*. 2010). *DHCR7* coding gene is located on chromosome 11q13.4. DHCR7 enzyme catalyses the production of cholesterol from dehydrocholesterol and can control the amount of available 7-dehydrocholesterol for previtamin D synthesis. *CYP2R1* coding gene is located in chromosome 11p15.2 and it encodes the enzyme vitamin D 25-hydroxylase in humans. It is a member of the cytochrome P450 enzyme family. The enzyme converts vitamin D_3 into 25(OH)D in the liver. A mutation in the *CYP2R1* gene leads to amino acid substitution and eliminates the enzyme activity and is associated with low circulating levels of 25(OH)D and vitamin D deficiency (Cheng *et al.* 2004). *CYP27B1* is a gene coding the 1α-hydroxylase enzyme that converts vitamin D to its active form 1,25(OH)D in the kidney and is located on chromosome 12q14.1. *CYP24A1* gene encodes the mitochondrial 1,25-dihydroxyvitamin D_3 24-hydroxylase enzyme. *CYP24A1* gene is located on chromosome 20q13.2 and takes part in the degradation of 25(OH)D and 1,25(OH)D (Jones *et al.* 2012). VDR gene encodes vitamin D receptor (Issa *et al.* 1998, Brown *et al*. 1999). VDR is a nuclear transcription factor that regulates the transcription of vitamin D target genes. It belongs to the nuclear hormone receptor superfamily (Bouillon *et al.* 1998, Issa *et al*. 1998). VDR is activated when a ligand 1,25(OH)D, is bound to it. Furthermore, VDR heterodimerizes with other nuclear receptors and binds to specific DNA sequences in the promoter region of vitamin D target genes (Saccone *et al.* 2015). *VDR* genes are expressed in almost all tissues in the human body and can regulate the expression of hundreds of genes (Bikle 2014).

2.2 Free 25(OH)D

Most of the circulating vitamin D metabolites are bound to DBP (85%) and approximately 15% to albumin. Only 0.4% of 1,25(OH)D and 0.03% of 25(OH)D is free in serum. Bioavailable 25(OH)D comprises of the free fraction and the fraction bound to albumin. According to the free hormone hypothesis, only hormones that are not bound to binding protein, such as vitamin D metabolites, sex steroids, cortisol, and thyroid hormone, are able to enter cells and have biological effects there (Mendel 1989). Vitamin D can enter the cells bound to DBP through megalin/cubilin receptor or through the cell membrane with passive diffusion of the free 25(OH)D (Nykjaer 1999). The concentration of free 25(OH)D is in equilibrium with the DBP-bound 25(OH)D, and the concentration of both bound and free forms are dependent on DBP and albumin concentrations. The in vivo studies in mice that lack DBP supported the hypothesis that free 25(OH)D and $1,25(OH)_2D$ concentrations are biologically more important than total concentrations. DBP-null mice maintained normal calcium homeostasis when fed a vitamin D-rich diet and had no evidence of bone disease despite having very low levels of circulating 25(OH)D and $1,25(OH)_2D$ (Safadi *et al.* 1999). When DBP was added to keratinocyte, monocyte, or osteoblast cultures, or to kidney homogenates or bone tissue cultures, it inhibited cellular uptake and actions of vitamin D metabolites (Chun *et al.* 2014). Several clinical studies have shown that estimated free and bioavailable 25(OH)D are more strongly associated with BMD (Powe *et al*. 2011), PTH, serum calcium (Bhan 2014), and osteoporosis (Al-Oanzi *et al*. 2006) than total 25(OH)D.

2.2.1 Calculated vs. measured value of free 25(OH)D

There are different methods for measuring free and bioavailable 25(OH) concentration. Many studies have used calculation of free 25(OH)D. The calculation of free and bioavailable 25(OH)D concentrations is based on a mathematical formula that takes into account total 25(OH)D, DBP, and albumin concentrations as well as their affinity constants (Bikle *et al*. 1986). The calculation has some challenges: the formula uses the measured concentrations of DBP and 25(OH)D and is therefore dependent on the accuracy of the measurement of both 25(OH)D and DBP concentrations. The results of DBP assays differ depending on the assay. In addition, it presumes that the interaction

between 25(OH)D and DBP variants is similar in vivo to in vitro binding affinity binding constants. Earlier studies that measured affinity binding constants of several DBP variants under different conditions obtained inconsistent results depending upon the buffering agent used (Chun *et al.* 2010, Powe *et al.* 2013, Johnsen *et al.* 2014).

Also, a direct two-step immunoassay for quantifying free 25(OH)D has been developed. This assay is dependent on the quality of the antibody used to bind the free 25(OH)D. The measurement of free 25(OH)D and 1,25(OH)D is difficult because of their very low concentrations and physico-chemical behavior. The assay recognizes $25(OH)D_2$ less well than $25(OH)D_3$ (approximately 77%) and may, therefore, underestimate the free $25(OH)D_2$, but under most situations in which the predominant vitamin D metabolite is $25(OH)D_3$, the data compare well with those obtained from similar populations using the centrifugal ultrafiltration assay (Malmström *et al.* 2017).

2.3 Parathyroid hormone

PTH is secreted by the parathyroid glands and metabolized in the liver and kidney. There are several circulating fragments of PTH: Intact PTH, N-terminal, 1-34 aminoacid polypeptide, mid-molecule, and C-terminal peptide. Intact PTH and N-terminal PTH are the biologically active forms (Potts *et al.* 2005). PTH has an important role in the regulation of calcium homeostasis in bone, kidney and the intestine. In bone PTH mobilizes calcium from the skeleton by activating osteoclasts and increases calcium absorption from the intestine. In the proximal renal tube of kidney, PTH activates the hydroxylation of 25(OH)D to 1,25(OH)D, which leads to an increase in the intestinal calcium absorption (Holick 2005). PTH also increases calcium reabsorption from the thick ascending limb of nephrons and facilitates the secretion of phosphorus through the kidneys. Calcium sensing receptor of the parathyroid glands detects when serum calcium concentration is low and stimulates PTH release or suppresses PTH release in response to high serum calcium. The calcium-sensing receptor is also expressed in several other tissues including renal tubular cells, where it regulates calcium reabsorption, and bone tissue and intestinal cells.

It is well established that PTH and 25(OH)D concentrations are inversely correlated and that PTH increases when 25(OH)D concentrations are below a certain value. PTH has

been shown to decrease when vitamin D-insufficient subjects are given vitamin D supplementation (Sai *et al.* 2011). High serum PTH concentrations can be caused by low dietary calcium, skeletal muscle wasting, primary or secondary hyperthyroidism, chronic kidney disease or inadequate vitamin D. Chronically elevated serum PTH concentrations impact bone density negatively by increasing osteoclast activity and the urinary excretion of phosphorus (Åkerström *et al.* 2005).

2.4 Vitamin D binding protein

DBP has been known as a group-specific component (GC) since 1959 (Hirschfeld *et al.* 1960). In 1975 it was identified by Daiger *et al.* (1975) as the vitamin-D-binding protein of human plasma. DBP has been found in several mammalian species and also in fish. It may be present in all vertebrates as a vitamin D carrier protein (Daiger *et al.* 1975, Constans *et al.* 1985, Constans *et al.* 1987). DBP is a polymorphic protein with a molecular weight of 52-59 kDa. Its structure resembles that of albumin and the α-fetoprotein gene family (Speeckaert *et al.* 2006). DBP is composed of three domains of a similar structure with a C-terminal truncation of the third repeat. The amino acid sequence is composed of 458 amino acids (Cooke *et al.* 1985, Cooke *et al.* 1991, Braun et al. 1993). The first domain between residues 35 and 49 has a α-helical structure and it binds vitamin D_3 ligands. An actin binding domain is located between residues 373 and 403 (White *et al.* 2000). The vitamin D binding site is composed of hydrophobic residues of helices 1e6 (amino acids 35e49). The binding site is located at the surface of DBP, whereas the vitamin D binding site of the vitamin D receptor is a closed pocket in the inner structure of the receptor (Verboven *et al.* 2002). The DBP structure is shown in Figure 2.

DBP transports 25(OH)D from the liver to the kidneys. In kidneys and other organs, 25(OH)D is converted into 1,25(OH)D. Approximately 85-90% of the circulating 25(OH)D and 85% of the $1,25(OH)_2D$ is bound to DBP (Bikle *et al.* 1986). Chylomicrons can also bind small amounts of vitamin D (Haddad 1995). DBP is principally expressed in hepatocytes and in smaller amounts in the kidney. It delivers vitamin D into cells by megalin-mediated endocytosis. In addition to serum, DBP is localized in several body fluids such as urine, breast milk, ascitic fluid, cerebrospinal fluid, saliva and seminal fluid and organs (brain, heart, lungs, kidneys, placenta, spleen,

testes and uterus). In previous studies, DBP concentrations have been shown to be low after liver diseases (Bikle 1985, Bikle 1986), nephrotic syndrome, and malnutrition. In addition, pregnancy and oestrogen treatment elevate DBP concentrations (Bouillon *et al.* 1981, Hagenfeldt *et al.* 1991, Rejnmark *et al.* 2006). The explanation for lower concentrations is probably reduced synthesis or increased loss of the protein (Speeckaert *et al.* 2014). DBP concentrations are also shown to vary by the degree of insulin resistance (Ashraf *et al.* 2014, Yousefzadeh *et al.* 2014). Season, vitamin D sterols, or other calciotrophic hormones (such as calcitonin or PTH) do not influence DBP concentrations (Speeckaert *et al.* 2006).

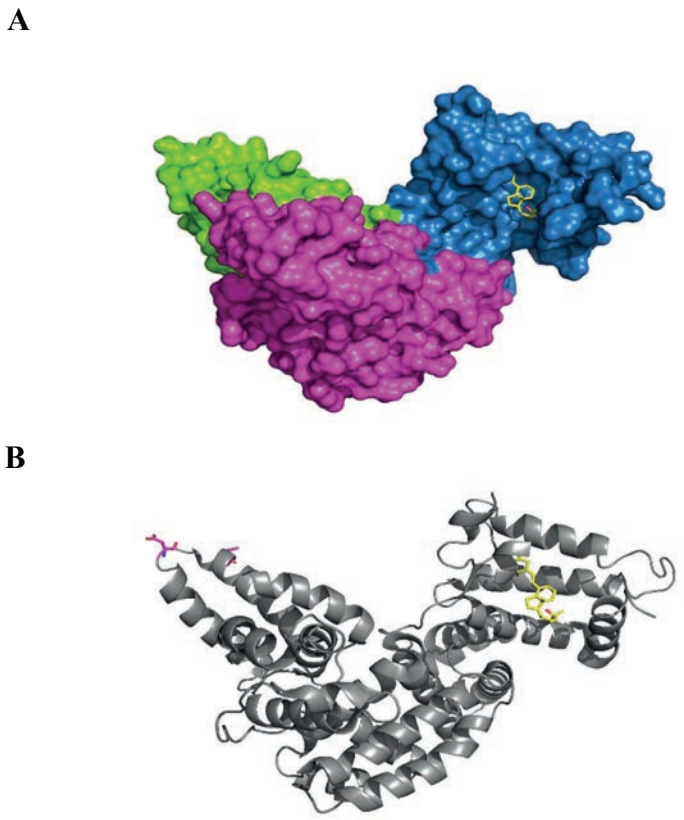

Figure 2 A three-dimensional representation of DBP. Surface representation (A) is showing the three domains in different colors (blue: domain I; purple: domain II; green: domain III) and the 25(OH)D shown in yellow stick model. α-helical structure is shown in figure 2 B and the common polymorphism sites (residues 416 and 420) are shown in pink. Figure is modified from protein data bank (PDB) structure 1J78 (Verboven *et al.* 2002).

2.4.1 Functions of DBP

The main function of DBP is to bind and transport vitamin D and its metabolites (Daiger *et al.* 1975). Each DBP-vitamin D metabolite complex has its own affinity constant. 25(OH)D binds DBP with a high affinity (K_a= 5×10^{-8} M), whereas 1,25(OH)D binds DBP with a lower affinity (K_a = 4×10^{-7} M) (Arnaud & Constans 1993, White *et al.* 2000). DBP has a high plasma concentration (0.32–0.46 g/L) and a short half-life (2.5–3 days) compared with 25(OH)D (5×10^{-8} M) and 1-2 months, respectively). Less than 5 % of DBP binding sites are occupied with vitamin D metabolites. The large molar excess of DBP in the circulation may protect against vitamin D intoxication by acting as a buffer when vitamin D concentrations rise or work as a circulating pool of 25(OH)D (Cooke *et al.* 1989, White *et al.* 2000). The functions of DBP are described in Figure 3.

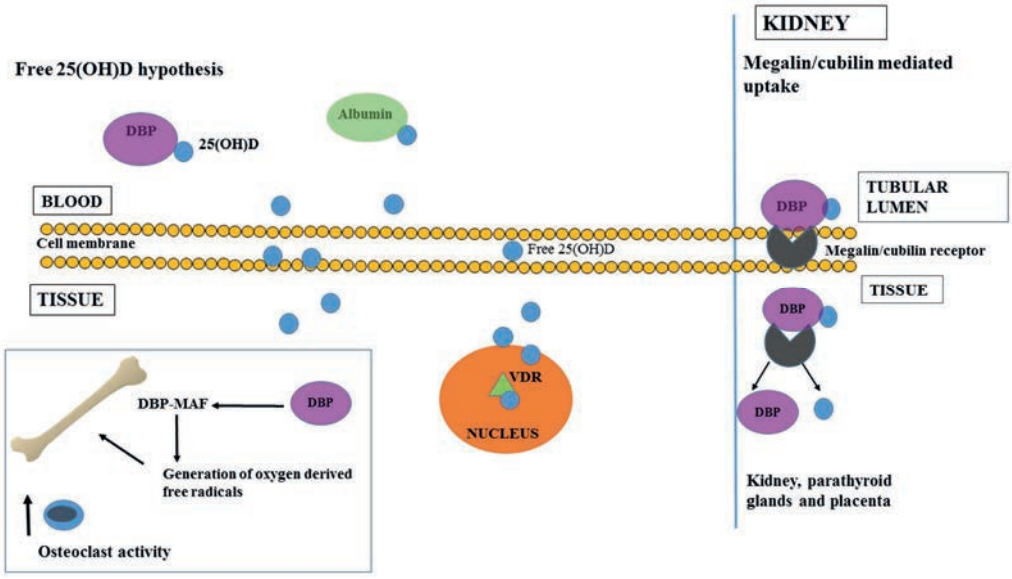

Figure 3. The free hormone hypothesis and the role of DBP-MAF in bone metabolism. Vitamin D metabolites (blue circles) are transported by DBP in the circulation and a small amount is also bound to albumin (bioavailable 25(OH)D) and lipoproteins. Free 25(OH)D and 1,25(OH)D can cross the cell membrane freely by diffusion and interact with the VDR. In many tissues e.g. the kidney, parathyroid and placenta, 25(OH)D and 1,25(OH)D are bound to DBP and enter the cells by endocytosis through the megalin/cubilin receptor. Modified from Delanghe *et al.* (2015) and Bikle *et al.* (2017).

2.4.2 Association of DBP with skeletal outcomes

Lower BMD is associated with vitamin D insufficiency. However, the links between DBP and bone biology are not fully understood. The importance of DBP for the skeletal system has been confirmed by disrupting the megalin gene in mice, resulting in an elevated urinary excretion of DBP, bone deformation and decreased bone density (van Hoof 2001). One mechanism for the effect of DBP on bone is through binding and transportation of vitamin D metabolites. Another mechanism may be acting through GC-macrophage activating factor (GC-MAF) (Yamamoto *et al.* 1993). GC-MAF is formed when DBP is deglycosylated by β-galactosidase and sialidase at the non-sterol binding domain, which involves the osteoclast activating domain (Haddad 1995, White *et al.* 2000). Several in vitro studies in mouse/rat peritoneal nonadherent cells have discovered that DBP is involved in macrophage activation. Furthermore, GC-MAF has been proposed to be a possible activator of osteoclasts. Osteopetrotic rats treated with GC-MAF showed a reduction in BMD and skeletal defects. In addition, osteopetrotic patients appeared to lack the ability to form GC-MAF (Yamamoto *et al.* 1994, Schneider *et al.* 1995, 2003). GC-MAF has also been suggested to activate osteoclasts by a cellular feedback mechanism that downregulates osteoclast activity when extracellular Ca concentrations rise (Yamamoto *et al.* 1996). GC-MAF can also influence calcium sensing of osteoclasts and thus stimulate bone resorption. The disruption of DBP receptor coding gene in mice resulted in elevated excretion of DBP in urine as well as deformation of bone and decreased bone density (van Hoof 2001).

2.4.3 Other roles of DBP

In addition to transportation of vitamin D and regulation the concentrations of free 25(OH)D available for utilization, DBP has also several other roles. Actin binds DBP with a high affinity and DBP has been suggested to be part of extracellular acting scavenging system. DBP can act as an extracellular scavenger for actin released from necrotic cells. DBP binds fatty acids, but compared to albumin the binding capacity is much weaker and there is a difference between them in the type of fatty acids they bind. Thus DBP has only accompanying role in fatty acid transportation. Poly-unsaturated fatty acids (arachidonic or linoleic acid) compete with vitamin D metabolites 25(OH)D and 1,25(OH)D for DBP binding. DBP has also been proposed to have a role in leukocyte C5a-mediated chemotaxis (Cleve 1988, Haddad 1995, Gomme *et al.* 2004, Speeckaert *et al.* 2006, Delanghe *et al.* 2015).

2.4.4 Measuring DBP

The specific method for measuring DBP has been under debate. DBP can be measured with ELISA-assay using monoclonal or polyclonal antibodies. There has been discussion about the suitability of measuring polymorphic DBP in groups of different skin colors and genotypes with a monoclonal method. The monoclonal assay may bind differently to DBP isoforms leading to underestimated concentrations. The underestimation occurs particularly in individuals with homozygous *GC*1F variant (Bikle *et al*. 2017). This genotype is more common in dark skinned individuals. In contrast to the monoclonal ELISA-assay, the polyclonal method is not subject to bias (Bouiollon *et al*. 2014, Denburg *et al*. 2016). A novel LC-MS/MS is also available for measuring DBP concentrations.

2.4.5 *GC* gene

DBP polymorphism has been established in both humans and primates (Constans *et al.* 1987). The human *GC* gene coding the DBP is localized on the long arm of chromosome 4 (4q12–q13). It over 35 kb long and contains 13 exons and 12 introns. The *GC* gene is a member of a multigene cluster. Albumin, α-fetoprotein, and α-albumin/afamin are other members of this cluster. All of these genes are primarily expressed in the liver (Speeckaert *et al.* 2006). The *GC* gene has over 240 different variants (76 synonymous, 155 missense and 11 loss of function). The *GC* gene has two well-known missense variations in exon 11: a G > A substitution at codon 416 (E416D), which leads to a glutamic acid to aspartic acid change, and a C > A substitution at codon 420 (T420K), which results in a threonine to lysine amino acid change (Braun *et al.* 1993) (Table 1). Haplotypes of these nucleotide changes form protein isoforms GC1S, GC2, and GC1F. Each *GC* gene variant has different combinations of two single-nucleotide polymorphisms (rs4588 and rs7041) resulting in two amino acid substitutions and differing glycosylation patterns. The two gene variants, rs4588 and rs7041, in general combine to form six diplotypes: *GC*1S/1S, *GC*1S/2, *GC*1F/1F, *GC*1F/2, *GC*1F/1S, and *GC*2/2 (Arnaud & Constans 1993). The structure of *GC* gene is shown in Figure 4 and the SNPs associated with vitamin D metabolism are indicated in the picture.

Table 1. Alleles of *GC* SNPs rs7041 and rs4588 and the resulting protein composition.

Variant	Alleles	Protein composition at 416 and 420
***GC*1F**	T/C	Aspartic acid/Threonine
***GC*1S**	G/C	Glutamic acid/Threonine
***GC*2**	T/A	Aspartic acid/Lysine

Polymorphisms in GC gene associated with 25(OH)D concentrations

The genetic polymorphisms in the two common SNPs (rs4588 and rs7041) of DBP have repeatedly been associated with 25(OH)D concentrations (Table 2). The protein isoforms differ in their binding affinity and capacity for carrying vitamin D metabolites. They also influence the plasma concentration of 25(OH)D and $1,25(OH)_2D$. The association of these SNP's with vitamin D concentrations has been found in several genome-wide association studies (GWASs) (Ahn *et al*. 2010, Wang *et al.* 2010, Sapkota *et al.* 2016, Jiang *et al.* 2018) and many candidate gene studies indicating that this polymorphism has the greatest impact on 25(OH)D concentrations of all vitamin D related polymorphisms.

Beside the two common SNPs, also other polymorphisms in the *GC* gene have been associated with 25(OH)D concentrations. Two GWASs in European subjects (Ahn *et al.* 2010, Wang *et al.* 2010) and two studies in Chinese subjects (Lu *et al.* 2012, Zhang *et al*. 2013) reported SNP rs2282679, to have an association with vitamin D deficiency. SNP rs1155563 is a GC gene variant that has been reported to be related to serum 25(OH)D concentration in several studies (Ahn *et al*. 2009, Lu *et al.* 2012, Hibler *et al.* 2014), and it has a high linkage disequilibrium (LD) with SNP rs4588. Two SNPs (rs16846876 and rs222020) were identified that are not in LD with rs4588 and rs7041 (Bu *et al.* 2010, Hibler *et al*. 2012). SNP rs2298849, located in the first intron, has been linked to serum 25(OH)D concentrations in an African American population (Signorello *et al*. 2011) and a population of Chinese post-menopausal women (Xu *et al*. 2014). Six SNPs in the GC gene (rs4588, rs16846876, rs2282679, rs12512631, rs17467825, and rs842999) were significantly associated with 25(OH)D levels in Danish populations. Of these, GC SNPs rs4588 and rs842999 were in strong LD. GC rs4588 and rs842999 without risk alleles had higher 25(OH)D concentrations than carriers of risk alleles (Nissen *et al*. 2014).

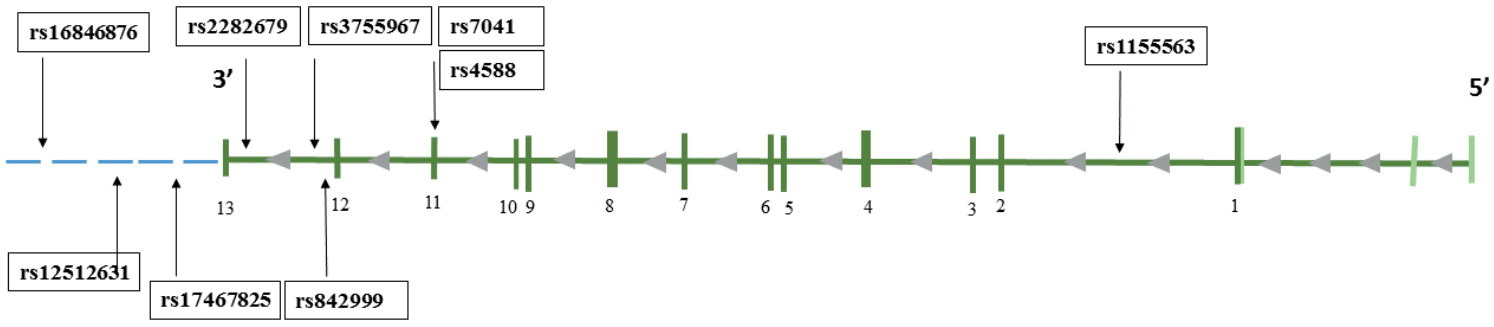

Figure 4 Structure of the *GC* gene. The gene consists of 13 exons, shown in the picture as dark green vertical lines. SNPs associated with vitamin D metabolism are indicated in the picture. The dashed blue line is an area outside the gene.

Table 2. Summary of reviewed studies on the association of *GC* gene SNP's with 25(OH)D concentrations

Reference	Subjects	*GC* SNP's	Main findings
Ahn *et al*. 2010	European ancestry from five cohorts, N=4501	GWAS	- SNPs rs2282679, rs7041 and rs1155563 were associated with 25(OH)D concentrations. -A heterozygote of SNP rs2282679 was at higher risk of vitamin D deficiency.
Wang *et al*. 2010	European ancestry from 15 cohorts, N=33996	GWAS	- SNP rs2282679 associated with 25(OH)D concentrations. -Participants with a genotype score in the highest quartile were at increased risk of having 25(OH)D < 75 nmol/L or < 50 nmol/L compared with the ones in the lowest quartile.
Sapkota *et al*. 2016	A Punjabi Sikh diabetic cohort, N=3538	GWAS	-Allele G of SNP rs2282679 was negatively associated with 25(OH)D concentration.
Jiang *et al*. 2018	Subjects of European descent, N=79 366	Meta-GWAS	- Common genetic variants regulated the 25(OH)D concentrations.
Ganz *et al*. 2018	Healthy third-trimester pregnant women, N=26 lactating women, N=28 non-pregnant women, N= 21	rs7041	- *GC* SNP rs7041 altered circulating vitamin D metabolites in all the study groups. - SNP rs7041 T allele associated with a different metabolic response to pregnancy and placental vitamin D metabolism.
Petersen *et al*. 2017	Healthy 8–11-year-old Danish children, N=642	rs4588 rs7041 rs2282679 rs842999	- rs4588 and rs7041 associated with lower 25(OH)D concentrations in all seasons.
Moon *et al*. 2017	White women (351 placebo, 331 cholecalciferol) double-blind, randomized, placebo-controlled trial of vitamin D supplementation in pregnancy.	rs2282679	- In the vitamin D supplement group, the common A allele of rs2282679 was associated with higher 25(OH)D concentration at 34 week pregnant women.
Zhang *et al*. 2013	Chinese subjects, N=2897.	rs2282679	- rs4588 and rs2282679 associated with 25(OH)D concentrations

Table 2 continues

Reference	Subjects	*GC* SNP's	Main findings
Li *et al*. 2017	A cross-sectional study of elderly post-menopausal Chinese women, N=967.	rs4588 rs7041	- SNP rs7041, but not rs4588, was associated with DBP concentration. - DBP correlated positively with 25(OH)D but negatively with bioavailable 25(OH)D. - Bioavailable 25(OH)D may be a more reliable biomarker in bone health.
Perna *et al*. 2013	Cohort study of 2160 women and 1581 men, aged 50-74 years.	rs4588 rs2282679 rs1155563	- Inverse association between rare alleles of SNPs rs4588, rs2282679 and rs1155563 and vitamin D concentrations. Association was stronger in summer months.
Nissen *et al*. 2014	Healthy Danish children and adults, N=758	rs4588, rs222020 rs842999 rs2882679 rs2298849 rs12512631 rs16846876 rs17467825	- SNPs rs4588, rs842999, rs2282679, rs12512631, rs16846876 and rs17467825 were associated with 25(OH)D concentrations in children, adults and, all combined. - rs4588 and rs842999 had significantly higher 25(OH)D concentration than carriers of all risk alleles.
Lu *et al*. 2012	Chinese Hans, N=3210	rs4588 rs7041 rs2282679	- *GC* SNPs rs4588, rs7041, rs2282679 and rs1155563 were all associated with lower 25(OH)D. - *GC* haplotypes that contained all risk-alleles (TACC) was significantly associated with lower plasma 25(OH)D. - Haplotype containing the risk alleles of rs4588 and rs2282679 (TATC) were associated with lower 25(OH)D.
Hibler *et al*. 2014	Ursodeoxycholic acid (UDCA) clinical trial subjects, N= 403	7041 4588	- Lower 25(OH)D concentration in clinical trial participants with 1F/2, 1S/2, or 2/2 compared with 1S/1S. - Cellular data revealed that 25(OH)D uptake varied less by *GC* isotype only at the higher concentration tested while 1,25(OH)D uptake differed markedly by *GC* isotype across concentrations and assays.
Hibler 2012	Participants from the Ursodeoxycholic Acid (UDCA) and Wheat Bran Fiber (WBF) trials N=404 subjects	rs7041 rs222035 rs842999 rs1155563 rs12512631 rs16846876 rs1746825	- Significant associations between *GC* SNPs rs7041, rs222035, rs842999, rs1155563, rs12512631, rs16846876, and rs1746825 and circulating 25(OH)D
Bu *et al*. 2010	Caucasian subjects, N=156.	9 genes in vitamin D metabolic pathways, 49 SNPs	- *GC* genotype may contribute to the variation of 25(OH)D in healthy populations.

Polymorphisms in GC gene associated with bone

GC polymorphisms have been linked with alterations in bone density in several populations. One of the mechanisms by which DBP may alter bone health involves regulating vitamin D bioavailability. Additionally, DBP may have independent roles in macrophage and osteoclast activation. The common GC SNP's are situated in exon 11(domain III) which is also an area responsible for nonsterol activities of DBP together with domain II and furthermore of the macrophage/osteoclast activation of DBP (Fang *et al.* 2009). Several studies have reported an association of *GC* polymorphism with bone density and fracture risk (Table 3). The results are inconsistent between the studies, and it is unclear whether there is an interaction of DBP or *GC* genetic polymorphism and circulating 25(OH)D concentrations and bone mass.

Table 3. Summary of reviewed studies on the association of DBP SNPs with bone traits

Reference	Subjects	SNP	Method	Main Finding
Ezura *et al.* 2003	Adult Japanese women, N=384	Multiple SNPs of the GC gene	DXA	-Multiple *GC* SNPs might contribute to increased risk of osteoporosis.
Lauridsen *et al.* 2004	Danish (white) menopausal women, N=595	rs4588, 7041	Bone density and bone mineral content were measured with DXA, history of fractures was collected	-DBP phenotype associated with fracture risk. -No association of DBP phenotype with bone mineral content or BMD. -DBP concentration correlated with fractures.
Fang *et al.* 2009	over 55 years old Caucasian men, N=3105 and women, N=4878	rs7041 rs4588	Fracture risk	- *GC* haplotypes associated with 25(OH)D concentrations. - *GC* gene had an effect on fracture risk, especially at low dietary Ca intake or in combination with an osteoporosis-risk allele of the VDR gene.
Xu *et al.* 2010	Caucasian men and women from United States, N=1873	12 SNPs of the *GC* gene	DXA	- *GC* SNPs rs222029 and rs222020 were associated with bone compression strength index in men.
Giroux *et al*. 2010	N=673 18–35-year-old women	12 SNPs of the GC gene		- No association of *GC* genotype with BMD.

Geographical distribution of the GC alleles

The GC SNPs rs4588 and rs7041 have a geographical distribution over three common alleles. Caucasian populations have a lower frequency of the *GC*1F allele and a higher frequency (50-60%) of the *GC*1S allele. The *GC*1F allele frequency is higher among black Americans and black Africans. The *GC*1F and *GC*1S allele frequencies have a geographical cline from Southeast Asia, through Europe and the Middle East, to Africa. In all populations, there is less of the *GC*2-allele than the *GC*1 allele, although Europeans do have a markedly higher GC2 allele frequency than do other ancestral groups. The variation in the *GC* allele frequencies may be correlated with skin pigmentation and intensity of sun light exposure. Dark-skinned and keratinized skin types have a lower rate of UV light penetration and a higher risk of rickets. The higher frequency of *GC*1F in dark- skinned persons relative to the white-skinned population may be due to its greater affinity for and more efficient transport of vitamin D metabolites (Malik *et al*. 2013). Several studies in black and white Americans and many other large genetic studies in numerous populations worldwide (Europeans, West Africans, Arabs, Danes, Korean, and Chinese) have shown associations between the *GC*1F and *GC*2 variants and lower concentrations of serum 25(OH)D (Constans *et al*. 1985).

2.5 Vitamin D, DBP, and obesity

Recent studies have suggested that there may be a connection between vitamin D deficiency and cardiometabolic diseases, such as obesity, impaired glucose tolerance, and diabetes mellitus type 2, arterial hypertension, and atherogenic dyslipidemia. Although the mechanisms are still unclear, vitamin D deficiency is associated with a greater risk of these conditions (Forman *et al.* 2008, Heaney *et al.* 2008, Bikle *et al*. 2009, Holick *et al.* 2011, Vanlint 2013, Ke *et al.* 2015, Wang *et al.* 2016, Gul *et al.* 2017, Lips *et al.* 2017).

Alterations in the vitamin D endocrine system have been shown to be associated with obesity. Increased adiposity is reported to be positively associated with serum PTH concentrations and inversely associated with serum 25(OH)D concentrations (Wortsman *et al*. 2000, Snijder *et al*. 2005, Shirazi *et al*. 2013). One reason could be the increased sequestration of 25(OH)D in excess subcutaneous fat, leading to a decrease in the bioavailability of vitamin D for calcium absorption (Drincic *et al*. 2012). The decreased availability of serum 25(OH)D causes an increase in PTH secretion to maintain serum

calcium concentrations. Other reasons suggested for lower vitamin D status in obese subjects could be low dietary intake of vitamin D and less sunlight exposure due to limited mobility or clothing habits among the obese compared with normal-weight individuals (Compston *et al*. 1981, Hypponen&Power 2007). Although the circulating 25(OH)D may be lower in obese individuals than in normal-weight peers, concentrations of vitamin D might not be lower and obese subjects may have greater vitamin D stores than normal-weight individuals. This in combination with low intake of vitamin D may lead to lower serum concentrations. However, overweight may also be the consequence of poor vitamin D status.

Many studies have investigated the relationship between 25(OH)D and insulin concentrations (Mc Gill *et al*. 2008, Pinelli *et al*. 2010). Vitamin D receptors and 1-α-hydroxylase have been found in pancreatic ß-cells, and this finding has led to studies on the possible effects of calcitriol on regulation of insulin production. Hence, vitamin D may have a role in controlling the function of ß-cells (Bland *et al*. 2004).

DBP has been shown to be lower in obese adolescents than in normal-weight subjects, and DBP and 25(OH)D correlated positively. There was also an inverse relationship between insulin and DBP concentrations in obese subjects. The authors suggested that insulin suppresses the production of DBP (Ashraf *et al.* 2014). In another study, obese women had higher DBP concentrations and lower free 25(OH)D than normal-weight women. The obese women were more likely to have 25(OH)D concentrations that could be considered suboptimal (Karlsson *et al*. 2014).

2.6 Bone

2.6.1 Bone structure

Bone comprises of cortical bone and trabecular bone. Up to 66% and 75% of lumbar and thoracic vertebrae is trabecular bone, respectively, whereas at distal radius only 5% is trabecular. The femoral neck is 75% cortical bone. Cortical and trabecular bone differ in their response to diseases, medication, hormonal changes, muscle-loading and impact-loading physical activity. Cortical bone is dense and solid tissue that surrounds the marrow space and trabecular bone. Cortical bone is located at the periosteal surfaces and in the middle of long bones and is responsible for the strength of the bone, provides resistance against bending and prevents bone from fracturing. Trabecular bone is a porous structure and consists of trabecular plates and rods, which strengthen the bone. Because trabecular bone is less dense, it has more surface area per unit volume than cortical bone and it is therefore flexible but weaker than cortical bone. Trabecular bone is located at the endosteal surfaces and at the end of long bones. Trabecular bone is metabolically more active and has a much higher remodelling rate.

Cortical bone is essential for the support of the body and its organs. Cortical bone also stores and releases chemical elements, most importantly calcium. Cortical bone consists of several microscopic columns (osteons), which are the functional units. Osteons have multiple layers of osteoblasts and osteocytes around Haversian canal. The osteons are connected together by Volkmann's canals. Nerves and blood vessels go through Haversian canals. Cortical bone is divided into periosteal surface and endosteal surface. Periosteum surrounds the outer cortical surface of the cortical bone and endosteum covers the inner surface and separates cortical bone and trabecular bone. Periosteum contains blood vessels, nerve fibres, osteoblasts, and osteoclasts. It protects and nourishes the bone and takes part in bone formation. Periosteum has an important role in appositional growth and fracture repair (Kini & Nandeesh 2012, Florensio-Silva *et al.* 2015).

2.6.2 Bone remodelling

Bone undergoes continuous remodelling. Remodelling helps bone to adapt to changes and to remove old bone and replace it with new stronger bone. Osteoclasts remove old bone by resorption and osteoblasts form new bone. Also the bone lining cells and osteocytes take part in bone remodelling. In growing bones, there is less remodelling and more modelling. During growth new osteons are formed and more bone is formed than resorbed. Modelling takes place mainly at the epiphysis of long bones leading to changes in shape and size of bone (Rauch *et al.* 2001, Bikle 2012). During ageing, bones expand in response to periosteal apposition of new bone and endosteal resorption of old bone. Bone modelling may increase due to hypoparathyroidism, renal osteodystrophy or treatment with anabolic agents (Ubara *et al.* 2003, Ubara *et al.* 2005, Lindsay *et al.* 2006).

2.6.3 Skeletal life span

Childhood and adolescence

Childhood and adolescence are characterized by longitudinal growth of bones as well as in changes in skeletal size and shape. Bone modelling begins already during foetal growth. Bone mass increases steadily during childhood and markedly during adolescence. By the end of adolescence, most of the adult skeleton is formed. Skeletal growth involves deposition and resorption of bone where bones expand (periosteal apposition of cortical bone) and lengthen (endochondral ossification) (Parfitt 1994). By the end of the second decade of life (19 years), epiphyseal fusion occurs (Heaney *et al.* 2000). Increases in trabecular bone in spine and long bones take place between sexual maturation stages (i.e. Tanner stages) III and IV. The density of cortical bone is lower among children and adolescents than among adults. Especially in boys, there can be occasional changes in cortical bone where the porosity of the bone increases. Boys have greater bone mineral content (BMC) and areal BMD than girls and the differences become stronger in puberty (Weaver *et al.* 2016). BMD increases during growth due to rise in bone size. However, volumetric bone density increases only modestly (Seeman 2002).

Adulthood

The adult skeleton is quite stable between the ages of 20 and 40 years and bone modelling is less frequent than remodeling in adults. Lifestyle factors, such as nutrition, smoking, alcohol use, and physical activity and, certain medications and diseases may have an impact on bone (Kobayashi *et al.* 2003).

In women, there is a reduction of bone at menopause due to a decrease in oestrogen production. Women are estimated to lose 30-50 % of their bone mass by the age of 70 years. In men, the bone loss is not so rapid. They have a steady continuous decline in bone mass in the fifth and sixth decade of life (40-60 years). Women are more susceptible to osteoporosis than men, due to their lower peak bone mass and greater bone loss.

2.6.4 Osteopenia and osteoporosis

Osteoporosis is characterized by lowered BMD due to accelerated bone turnover. In this condition, more bone is resorbed than formed. These changes lead to bone fragility and predisposition to fractures. In osteoporosis the amount of bone is decreased and the structure of trabecular bone is impaired. In addition, cortical bone becomes more porous and thinner, making the bone weaker and more prone to fracture (Karaguzel & Holick 2010, Mirza *et al.* 2015). Bone loss occurs in all humans due to hormonal changes. The primary reason for both sexes is estrogen deficiency. Osteopenia is a precursor phase to osteoporosis but it does not always develop into osteoporosis. The difference between osteopenia and osteoporosis is based on BMD. Both men and women have a risk of having osteopenia/osteoporosis, but menopausal women are at the highest risk. Osteoporosis is divided into primary and secondary osteoporosis. Osteoporosis is classified as primary, when it occurs in postmenopausal women and men and is age-related and there is no underlying disease. Secondary osteoporosis is a condition that occurs when there is an underlying disease or medication (Mirza *et al.* 2015).

T-score and Z-score

T-score is often used when bone density measurements are interpreted. T-score is the number of standard deviations below or above the average healthy adult bone mass. T-scores depend on the reference group used (e.g. Caucasian women or Hispanic men). Z-score is the number of standard deviations below the average of the same-age and gender person. There are also different Z-scores depending on the group used as a reference. T-score and Z-score can be calculated for each bone parameter. In osteopenia BMD T-score is between −1.0 and −2.5. In osteoporosis the T score is lower than −2.5. The WHO definition is based on BMD measurement with DXA at the spine, hip, or forearm (Kanis *et al.* 1994).

2.6.5 Biochemical markers of bone metabolism

Bone formation and resorption rates can be detected with several biomarkers from serum or urine. The markers are often bone proteins, collagen fragments, peptides, or enzymes. Alkaline phosphatase (ALP) is important for bone mineralization and both total ALP and bone specific ALP (BALP) are used as bone formation markers. Other bone formation markers include bone matrix protein osteocalcin and propeptides of collagen I called carboxy-terminal and amino-terminal propeptides of type I procollagen (PICP and PINP).

Markers of bone resorption, in turn, include, collagen degradation products: telopeptides of type I collagen: Carboxy-terminal Crosslinked Telopeptide of type I collagen (CTX-I) and CTX-matrix metalloproteinases and N-terminal telopeptide I (NTX-I), Hydroxyproline, Deoxypyridinoline, Pyridinoline and Hydroxylysine. Non-collagenous proteins include Bone sialoprotein (ostepontin), and osteoclastic enzymes: Cathepsin K and Tartrate-resistant acid phosphatase. Bone turnover regulators consist of Receptor activator of nuclear factor kappa-B ligand (RANKL), Osteoprotegerin (OPG), Dickkopf related protein 1, and Sclerostin (Kuo & Chen 2017).

Osteocalcin

Osteocalcin (bone gamma-carboxyglutamic acid-containing protein) is a small protein synthesized by mature osteoblasts, odontoblasts, and hypertrophic chondrocytes. The synthesis of osteocalcin is stimulated by 1,25(OH)D. Most of the osteocalcin is situated in bone. Carboxylation of glutamic acid residues leads to a conformational change in protein. The change in protein structure allows the binding of osteocalcin with hydroxyapatite and mineralization in the bone matrix (Hlaing *et al.* 2014). Osteocalcin is also secreted in the circulation. The protein has an important role in metabolic regulation, bone mineralization and calcium homeostasis (Lee *et al.* 2007).

Serum osteocalcin has been used as a marker of bone formation rate in osteoporosis. Postmenopausal women with osteoporosis or osteopenia have been shown to differ significantly in their osteocalcin concentrations from non-osteoporotic women. Consequently, osteocalcin may be useful for determining osteoporosis in elderly persons and especially in women (Singh *et al.* 2015).

Procollagen type I N-terminal propeptide (PINP)

PINP is synthesized by fibroblasts and osteoblasts and is an indicator of type I collagen deposition in bone matrix. Procollagen type I has N-terminal and C-terminal extensions, which are removed by specific proteases when it is converted to collagen. PINP is released during formation of type I collagen into the intracellular space. PINP concentrations can be measured by ELISA-assay or radioimmunoassay using PINP antibodies. PINP has been demonstrated to be a sensitive biomarker to measure bone formation rate in osteoporosis (Kuo & Chen 2017).

Carboxy-terminal crosslinked telopeptide of type I collagen

Telopeptides of type I collagen are bone resorption biomarkers that include collagen type I carboxy-terminal crosslinked (CTX-I) and amino-terminal crosslinked (NTX-I) telopeptides. They are both released during collagen degradation. NTX and CTX are excreted in urine and can also be measured from serum. The concentrations have circadian

variation and therefore the timing of the sample collection is important. CTX and NTX can be measured with ELISA, RIA and electrochemoluminescence assays. The advantage of serum markers compared to urine markers is that the results do not need correction with creatinine (Hlaing *et al.* 2014, Kuo & Chen 2017).

2.6.6 Factors affecting bone

Vitamin D

Vitamin D deficiency is one factor that predisposes bone to osteoporosis. Vitamin D deficiency causes a decrease in calcium absorption, which is compensated by an increase in PTH, stimulating the conversion of 25(OH)D into $1,25(OH)_2D$. In vitamin D deficiency, calcium absorption decreases leading to increase in PTH. Excessive secretion of PTH by the parathyroid glands called secondary hyperparathyroidism, in response to low blood calcium concentrations, increases bone turnover, leading to bone loss (Lips 2001, 2006). Severe vitamin D deficiency can result in rickets in children or osteomalacia in adults. Studies have found a positive association between 25(OH)D concentrations and BMD (Outila 2001, Bischoff-Ferrari 2004). There is convincing evidence that supplementation with vitamin D at a dose of 10–20 µg/d combined with calcium, reduces the risk of total fracture and hip fracture. The effect is more pronounced in institutionalized elderly persons, and supplementation with vitamin D alone has not shown a significant impact (Lamberg-Allardt *et al.* 2013).

Other determinants of bone health

Lifestyle factors, such as nutrition and physical activity, are important for bone mass accrual during growth and for maintaining bone in adulthood. The role of calcium and vitamin D in improving BMD and reducing fracture risk has been established. Persistently low 25(OH)D may result in prolonged elevation of PTH, leading to an increase in bone resorption. The optimal concentration of 25(OH)D required for optimum peak bone mass remains to be

elucidated. Adequate protein intake is also important for bone health. Excessive intake of alcohol has adverse effects on bone-forming cells as well as on the hormone that regulates calcium metabolism. Also poor nutritional status (calcium, protein, and vitamin D deficiency) due to excessive alcohol consumption can cause secondary osteoporosis (Kanis *et al.* 2005). People with a past history of smoking and current smokers are at higher risk of having osteoporosis than non-smokers (Kanis &Johnell 2005). Eating disorders, such as anorexia nervosa and bulimia, also affect bone negatively. Oestrogen deficiency in post-menopausal women accelerates bone loss. Certain medications such as corticosteroids can affect bone negatively. Osteoporosis induced by glucocorticoids is primary cause of secondary osteoporosis (Mitra 2011). Genetic factors play an important role in the pathogenesis of osteoporosis. It has been suggested that up to 50-85% of peak bone mass and some geometrical properties can be determined genetically (Ralston 2006).

2.6.7 Bone assessment

Dual energy X-ray absorptiometry (DXA)

DXA measurements are used in accordance with a WHO guideline for the diagnosis of osteoporosis and fracture risk in adults. DXA measures the attenuation of X-rays of two different energies when passed through the body, and computes the BMC (in grams) and areal BMD for a given region. The common measurement sites for DXA are hip and lumbar spine. Also whole-body BMD measurement is used. The limitation of DXA is that it gives only a two-dimensional image and cannot, therefore, separate cortical and trabecular bone, or assess three-dimensional geometry and microarchitecture (Bouxsein 2008).

Peripheral quantitative computed tomography (pQCT)

Peripheral quantitative computed tomography (pQCT) is used for imaging bone as well as fat and muscle tissues surrounding the bone. pQCT is used only for research purposes, not in clinical practice. pQCT gives a three-dimensional image of the bone. Trabecular- and cortical- and volumetric bone density (vBMD) as well as bone size and geometric properties, e.g. total cross-sectional bone area, cortical bone area, periosteal and endosteal

circumferences, and cortical thickness can be measured with pQCT from peripheral sites. The cross-sectional moment of inertia (CSMI) and other measures of bone strength, such as the bone strength index (BSI), polar strength strain index (pSSI), and cortical strength index (CSI), can then be calculated (Siu *et al.* 2003).

The benefits of pQCT compared with DXA are the very low radiation dose and the ability to distinguish between trabecular and cortical bone. pQCT-measurement also takes into account the size of the bone, and therefore, quantification of volumetric BMD is more reliable (Binkley *et al*. 2008).

3 AIMS OF THE STUDY

The purpose of this thesis was to investigate the associations of DBP genetic polymorphism with calcium homeostasis and BMD in children and adolescents as well as in middle-aged individuals in Finland. Specific aims were to examine the metabolism of free vitamin D and to determine whether obese individuals have altered vitamin D metabolism. The questions seeking answers in Studies I-III were as follows:

Study I: Does genetic polymorphism of DBP have on effect on BMD and calcium metabolism in Finnish children and adolescents in a cross-sectional setting?

Study II: Do total, free and bioavailable 25(OH)D, vitamin D binding protein, and PTH concentrations relate to the genetic variation of the *GC* gene in Finnish middle-aged women and men in a cross-sectional setting?

Study III: Do obese individuals have altered vitamin D metabolism and whether this is associated with BMD in a cross-sectional study of Finnish middle-aged women and men?

4 SUBJECTS AND METHODS

4.1 Subjects and study design

4.1.1 Study in children and adolescents (I)

Study I combined subjects from two different cross-sectional studies (the Optiford-study and the School-based study) in the capital region of Helsinki (60°N), southern Finland. The total study population consisted of 233 children and adolescents, 160 girls (mean age 13±2.5 years) and 73 boys (mean age 12.6±2.7 years). Participants in the Optiford-study were girls (N=50) aged 11-12 years and in the School-based study boys (N=73) aged 12.6±2.7 years, and girls (N=110) aged 13±2.5 years. The original purpose of the Optiford-study was to examine the efficacy of vitamin D-containing, dietary-supplement use in children and adolescents and the School-based study investigated vitamin D status and its association with vitamin D intake and bone health in children and adolescents. Recruitment in both studies was conducted in primary schools and in the School-based study also in a secondary school. School classes were randomly selected to the study. All participants were healthy and used no medication known to affect calcium metabolism. Over 90% of the subjects were Caucasian. The Optiford-study was conducted between September 2001 and March 2002 and is described in detail in Viljakainen *et al.* (2006). The School-based study was conducted during 2007-2008 and is described in detail in Pekkinen *et al.* (2012).

4.1.2. Cross-sectional study in middle-aged men and women (II-III)

The subjects in Studies II and III were 37- to 47-year-old Caucasian females and males from the study "Dietary phosphorus and health outcomes (PHOMI)", which is a cross-sectional study of Caucasians living in the Helsinki area (60°N, southern Finland) who were derived from the Population Register Centre. Recruitment and the study protocol are described in detail in Itkonen *et al.* (2013). The principal aim of the PHOMI-study was to examine

dietary phosphorus intake and its impact on cardiovascular and bone health. The study aimed to have 800 subjects (400 women, 400 men). The sample size was based on a statistical power of 80% (α= 0.05) to find a 4% difference (SD=0.050 g/cm^3) in distal radius trabecular density (TraD) between the highest and lowest phosphorus intake tertiles by analysis of variance. Initial dropout of 40% (based on 1200 participants) was taken into account. Pregnancy was an initial exclusion criterion in the study. Subjects visited the research unit two times during spring 2010: on the first visit (January or March), fasting blood samples and urine samples were collected and subjects were instructed to fill in a study questionnaire. The total number of participants in blood sampling was 678. In the second phase i.e. pQCT bone measurements, 653 subjects of these participated. Due to missing data (e.g. background, blood sample, genotype, DBP concentration), the total number of subjects included in Study II was 622 (421 women and 201 men). Morbidly obese subjects (BMI≥40), chronically ill and participants with frequent use of sunbed (> 10 times during 2008–2010) were excluded from the analysis. In total, 606 subjects were included in the Study II analysis. Also in Study III, morbidly obese subjects (BMI≥40), chronically ill and participants with frequent use of sunbed (>10 times during 2008–2010) were excluded from the analysis. In vitamin D, PTH and DBP analysis of Study III, the number of subjects was 548 (384 women and 164 men).

In Study III, subjects with a history of eating disorder, menopause (women who reported their menstrual cycle had ended), or medication or illnesses affecting calcium or bone metabolism were excluded from the pQCT-bone analysis resulting in 547 subjects (350 women and 197 men).

4.1.4 Ethics statement

All subjects in Studies I-III, and in Study I also the parents of the participants, gave their written informed consent to the study procedures. All of the studies were conducted in accord with the Helsinki Declaration. The study protocol was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa.

4.2 Methods

4.2.1 Background characteristics

Children and adolescents (Study I)

The subjects together with their parents completed a questionnaire on medical and fracture history, medications, overall health, age at menarche, use of vitamin D and Ca supplements, and details about their physical activity. A FFQ based on Outila *et al.* (2001) was used to measure dietary intakes of vitamin D and Ca during the previous month. Necessary changes were made to the FFQ to take into account changes in vitamin D-fortified products on the market. An updated version of the FFQ was later validated (Itkonen *et al*. 2016).

Weight was transformed into height-adjusted values (percentages of the mean in a normal population of the same sex and height), according to Finnish standards (Sorva 1990, Pere 2000). Pubertal development was scored as pre-, mid-, or postpubertal (School-based study) by a pediatric endocrinologist (Outi Mäkitie) or according to Tanner stages I–V (Optiford-study). The scoring was made based on questionnaire data and serum gonadotrophin and sex steroid concentrations. The Tanner stages were then transformed into pre-, mid-, or postpubertal categories to acquire a united scale for all subjects. Tanner stages I–II were considered as prepubertal, stages III and IV midpubertal and stage V as postpubertal. Physical activity consisted of everyday activities (e.g. walking to school), activity at school, and both guided and unguided leisure-time activities. Physical activity score was calculated by summing a whole week's different activities, as described in Pekkinen *et al.* (2012).

Adults (Study II, III)

The subjects answered a questionnaire on medications and overall health, use of vitamin D and Ca supplements, and details about their physical activity. A FFQ based on Outila *et al.* (2001) was used to measure dietary intakes of vitamin D and Ca during the previous month in all studies. Necessary changes were made to the FFQ to take into account changes in vitamin D-fortified products on the market. An updated version of the FFQ was later validated (Itkonen *et al.* 2016).

In Studies II and III, both supervised and unsupervised exercise as well as functional exercise were taken into account when physical activity was calculated in minutes per week. Sunshine exposure was estimated from the number of holidays spent in sunny locations (exposure to UV-irradiation) from November 2009 to January 2010 or from November 2009 to March 2010 depending on the month that blood was sampled. Amount of smoking was estimated as pack years (number of cigarette packs per day x number of years smoking). Weight and height were measured in light clothing and BMI was calculated as kg/m^2. In Study III, subjects were classified into BMI groups: normal-weight (18.5-24.9 kg/m^2), overweight (25-29.9 kg/m^2), or obese (30-39.5 kg/m^2) (WHO 2000).

4.2.2 Laboratory analyses

Blood samples and second void urine samples were collected at 8–10 am after an overnight fast between November and March (School based study) or between September and March (Optiford study). In the PHOMI study (Studies II and III), twelve-hour fasting blood samples and second void urine samples were collected between 7:30 am and 9:15 am in January or March 2010. A summary of the laboratory measurements in Studies I-III is provided in Table 4.

Table 4. Summary of laboratory measurements in Studies I-III

	Study	Method	Location of analysis	CV %
25(OH)D	I	HPLC (DEQAS)	DFVR (Optiford) HUS (School based-study)	Optiford: 4.3, 6.3 School based study: Information not available
	II, III	IDS enzyme immunoassay kit (Immunodiagnostics Systems Ltd., Boldon, UK)	UH	2.7 (inter) 3.2 (intra)
PTH	I	Commercial two-site immunoluminometric method	UH (Optiford), HUS (School based-study)	8.0 (inter), <5.5 (intra) (II, III)
	II, III	Immunoluminesence-based method (Immulite1000, Siemens Healthcare Diagnostics, NY, USA)	UH	
Calcium	I-III	Photometric method with Konelab20 automatic analyzer (Thermo Clinical Labsystems, Espoo, Finland)	UH (Optiford), HUS (School based-study) UH (II, III)	<4.6 (II, III)
Albumin	II, III	Photometric method with Konelab20 automatic analyzer	UH	<4.6 (II, III)
DBP	II, III	ELISA-assay using polyclonal antibodies	UH	
Osteocalcin	II, III	Two-site immunoassay-method based on monoclonal antibodies	Department of Cell Biology and Anatomy, Institute of Biomedicine, University of Turku	
S-CTX	II, III	IDS-iSYS Multidiscipline Automated Analyser	NordLab Oulu, Department of Clinical Chemistry, University of Oulu	intra<5.3, inter<2.9
S-iPINP	II, III	IDS-iSYS Multidiscipline Automated Analyser		
U-Ca, UPi, UCrea	I	Standard methods	UCC(Optiford) HUS (School based-study)	

DFVR; Danish Institute for Food and Veterinary Research, Søborg, Denmark, UCC; Department of Food and Nutritional Sciences and Biosciences Institute, University College Cork, Ireland, UH; Department of Food and Environmental Sciences, Division of Nutrition, University of Helsinki, Finland,
HUS; Central Laboratory of Helsinki University Central Hospital, Finland.

4.2.3 Genotyping (I, II)

Genomic DNA was extracted from whole-blood samples with commercially available kits. DNA extraction and genotyping methods are described in Table 5. In Study I, the rs4588 and rs7041 SNPs were genotyped. In Study II, SNPs covering the DBP gene region were selected from the International HapMap Project database (https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/).
The function "Download tag SNP data" was used and three polymorphisms were chosen: rs4588, rs7041and rs705124. These polymorphisms represented the linkage disequilibrium structure of a region. Preference was given to polymorphisms with high heterozygosity levels and SNPs that are functional and have previously shown association in other studies. Northern Europeans from Utah (CEU population) was used as a reference when selecting these three SNPs.

Table 5. DNA extraction and genotyping methods in Studies I-II

	Study	Method	
DNA extraction method	I	Optiford study: Wizard® Genomic DNA Purification kit, (Promega, Madison,WI, USA) School based study: Gentra Puregene Kit (Qiagen GmgH Hilden, Germany)	
	II	Qiagen DNA Mini Kit	
Genotyping	I	RT PCR Mx3000P, (Stratagene, La Jolla, CA)	**Primers:** 5′GGCAAAGTCTGAGCGCTTGTTA3′ and 5′CAGACTGGCAGAGCGACTAAAAG3′ **Probes:** 5′NEP/TTGCCT GAG GCC ACA CC/NEP-3′ 5′MAR/TTGCCTGATGCCACACCC/MA R-3′, 5′NEP/CCACACCCAAGGAACTGGC/NE P-3′ and 5′MAR/CACACCCACGGAACT GGC/MAR-3′
	II	TaqMan chemistry, an automatic sequence-detection instrument (ABI Prism 7900HT, Applied Biosystems, Foster City, CA, USA) Probes (AllGlo™, Allelogic Biosciences Corporation, Hayward, CA, USA).	**Probes:** FAM dye label VIC dye label SNP Assay ID C___8278879_10 SNP Assay ID C___3133594_30 SNP Assay ID C___8278822_10

4.2.4 Calculation of free and bioavailable 25(OH)D (II-III)

Calculation of free 25(OH)D in studies II and III was performed using the following equation where [F] is concentration of free 25(OH)D and [T] is concentration of total 25(OH)D and KALB and KDBP are the affinity constants for 25(OH)D with albumin and DBP and [ALB] and [DBP] are the concentrations of albumin and DBP (Bikle *et al*. 1986):

$$[F] = \frac{(T)}{(1 + KALB\,[ALB] + KDBP[DBP])}$$

GC haplotype and diplotype-corrected free 25(OH)D concentrations were calculated using diplotype specific binding constants according to Powe *et al.* (2013) and Johnsen *et al.* (2014), respectively (Table 6).

Table 6. Affinity constants between 25(OH)D and albumin, DBP, and different *GC* haplo-and diplotypes.

Affinity constants	
Albumin	6×10^5
DBP	7×10^8
Haplotype GC 1S	0.60×10^9
Haplotype GC 1F	1.12×10^9
Haplotype GC 2	0.36×10^9
Diplotype GC 1S/1S	6×10^8
Diplotype GC 1S/2	4.8×10^8
Diplotype GC 1F/1F	11.2×10^8
Diplotype GC 1F/1S	8.6×10^8
Diplotype GC 1F/2	7.4×10^8
Diplotype GC 2/2	3.6×10^8

4.2.5 Skeletal measurements

In Study I, BMD, BMC and bone area were measured with DXA (Hologic Discovery A, pediatric software, version 12.4) from the lumbar spine (LS) (L1-L4), total hip, and whole body (WB).The measurement protocol is described in detail in Pekkinen *et al.* 2012. Volumetric BMD and bone geometry were measured proximally at 4% and 66% from the distal end of the non-dominant radius with pQCT (XCT-2000; Stratec, Pforzheim, Germany) in a group of 175 subjects (104 girls, 71 boys). Total, trabecular, and cortical bone were measured and BMC, total bone (TB), cross-sectional area (CSA), and SSI were calculated. The DXA values were also transformed into Z-scores by using equipment-specific age- and sex-adjusted reference data for US Caucasian children and in pQCT according to references reported in the papers of Rauch *et al*. (2005) and Rauch & Schoenau (2008).

In Study III the distal sites of the non-dominant radius and left tibia were scanned at 4% and 5% from the distal endplate, respectively. The shaft sites of the radius and tibia were scanned at 30% from the distal endplate. The measurement protocol is described in detail in Laaksonen *et al.* (2010). The measured bone parameters were trabecular density (TraD) from the distal radius and tibia and cortical density (CorD) from the radial and tibial shaft sites. Furthermore, cortical strength index i.e. ratio of cortical bone area to total bone area (CSI=CorA/TotA) was calculated. The *in vivo* coefficients of variation (CV %) for the radius were 1.6% for the TraD at the distal site and 3.2% for the CorD 0.5% at the shaft site. The corresponding CV%s for the tibial traits were 0.5% and 0.6%.

4.3 Statistical analyses

The data are reported as means with standard deviations (SDs) or standard errors (SEs). Hardy–Weinberg equilibrium of the GC genotypes in Studies I and II was tested with Chi-square test. In Study I, association of variables was tested with Pearson correlation and if outliers were detected, Spearman correlation was used instead. Linear regression analyses were performed to determine the factors associated with bone traits measured with DXA and/or pQCT in children and adolescents using GC genotype and other selected independent variables. The relationship between GC genotypes and PTH was analysed only in the School based study to avoid bias of different PTH assays in the two groups (Optiford and School based study). Partial correlation was used to demonstrate the association after controlling for confounding factor(s). Associations between *GC* genotype, 25(OH)D and PTH were

analysed with analysis of covariance (ANCOVA) with relevant covariates. Test for linear trend was performed by using contrast analysis. Simple regression analysis in the whole study population was first performed to screen potential predictors for bone traits and a multivariate linear regression model was used to identify significant predictors. In regression analysis, *GC* genotype was used as a dummy variable (grouped into 0 (*GC 1/1*) and 1 (*GC 1/2*, *GC 2/2*)).

In Study II ANCOVA analysis was used to test variation among the *GC* genotypes in total, free, bioavailable, and genotype-adjusted 25(OH)D concentrations using relevant covariates. Also the variation between the *GC* genotypes and DBP concentrations and between the *GC* genotypes and PTH were tested with ANCOVA. Tests for linear trend among the genotypes in the 25(OH)D, PTH, and DBP concentrations were conducted using contrast analysis. In this analysis, the three genotypes of the SNP 4588 were coded as −1, 0, and 1.

In Study III, the correlation between variables of interest was tested with Pearson correlation. Background characteristics between the three BMI groups were compared with ANOVA. The difference between the BMI groups in total, free and bioavailable 25(OH)D, PTH, and DBP concentrations was tested with ANCOVA using relevant covariates. Post-hoc comparisons were made with Bonferroni correction. To determine the factors associated peripheral bone traits, a backward regression analysis was performed for men and women separately using selected independent variables. The model with the largest adjusted coefficient of determination (R^2) is presented in the results. Only the bone traits independent of body size were used in the analysis.

Statistical analyses were conducted with PASW 18.0 (Study I), PASW 18.0.2 (Study II) and SPSS24 (Study III). P-value<0.05 was considered significant.

5 RESULTS

5.1 Background characteristics of subjects (I-III)

5.1.1 Children and adolescents (I)

Baseline characteristics in girls and boys in three GC genotype groups are presented in Table 7. The relative weight of the subjects was above 20% (=overweight) in 31 subjects and above 40% (=obese) in 7 subjects. Vitamin D and Ca intakes were in line with the recommendations at the time (Nordic Nutrition Recommendations 2004) but compared with the current recommendations of ≥ 10 µg/d (Nordic Nutrition Recommendations 2012), vitamin D intake was lower than recommended in girls. Of the study population, 67% had 25(OH)D concentration below 50 nmol/L (68% of girls, 69% of boys). There was a negative correlation between 25(OH)D and month of sampling: the 25(OH)D concentrations were higher later in autumn than early in spring ($r=-0.184$, $P=0.005$), PTH correlated negatively with 25(OH)D ($r=-0.166$, $P=0.022$) after controlling for Ca intake.

Table 7. Baseline characteristics stratified by sex and *GC* genotype (I).

	Girls				**Boys**			
Genotype	***GC*1/1**	***GC*1/2**	***GC*2/2**		***GC*1/1**	***GC*1/2**	***GC*2/2**	
N	105	46	9	P	54	15	4	P
Age (years)	12.9 (2.3)	13.0 (2.6)	14.3 (3.9)	0.258	12.6 (2.7)	12.6 (2.2)	12.6 (4.8)	0.999
Height (cm)	154.8 (11.2)	154.3 (10.9)	153.0 (12.5)	0.881	154.8 (17.6)	155.8 (17.4)	157.7 (29.4)	0.946
Weight (kg)	46.3 (12.3)	47.1 (12.2)	42.9 (10.9)	0.637	48.9 (18.7)	48.6 (18.2)	45.3 (22.3)	0.931
Weight (%)	4.66 (17)	7.95 (15.6)	-1.11 (9.0)	0.267	8.17 (14.5)	9 (28.1)	-7.33 (0.6)	0.345
Pubertal stage (%)								
Prepubertal (N)	34	15	4	0.798	27	9	2	0.608
Pubertal (N)	35	15	0		9	3	0	
Postpubertal (N)	36	16	5		18	3	2	
Vitamin D intake (µg/d)	8.3 (4.7)	8.1 (5.6)	9.7 (6.9)	0.739	11.1 (5.4)	12.9 (5.7)	8.3 (2.0)	0.427
Calcium intake (mg/d)	1486 (622)	1328 (597)	1266 (509)	0.239	1602 (528)	1638 (456)	955 (125)	0.206
25(OH)D (nmol/L)	46.0 (15.4)	42.3 (12.4)	38.5 (9.0)	0.153	45.1 (12.8)	40.7 (9.5)	45.3 (8.0)	0.459
PTH (ng/L)	43.9 (22.4)	39.1 (13.0)	32.0 (8.5)	0.124	41.6 (24.2)	45.9 (17.8)	40 (18.3)	0.790
Physical activity score	16.5 (4.1)	15.9 (3.8)	13.7 (4.3)	0.202	14.8 (3.6)	15.4 (4.1)	14.5 (2.3)	0.870

ANOVA; values are shown as means (± SD).

5.1.2 Adults (II)

Background characteristics of subjects according to GC genotypes in Study II are presented in Table 8. The mean total vitamin D intake was 15.1 µg/d and intake from supplements was 7.2 µg/d. The mean Ca intake was 1253 mg/d and intake from supplements 87 mg/d. The daily vitamin D and Ca intakes (including supplements) were in line with Nordic recommendations (Nordic Nutrition Recommendations 2012). Women and men differed significantly in height, weight, serum albumin concentrations and BMI (P<0.001-0.002). There was also a difference between genders in history of smoking and amount of physical activity (P=0.013 and P<0.001). The mean 25(OH)D concentration in the total population was 56 nmol/L and the mean PTH concentration 54.6 ng/L. The mean DBP concentration was 368 mg/L. There was a negative correlation between PTH and total 25(OH)D (r=−0.235, P < 0.001). Negative correlations were also observed between free and bioavailable 25(OH)D and PTH (r=−0.223, P< 0.001; r=−0.217, P< 0.001). Total 25(OH)D correlated negatively with BMI (r=−0.173, P<0.001).

Table 8. Background characteristics of subjects according to *GC* genotypes (II).

Variable	***GC*1/1**	***GC*1/2**	***GC*2/2**	
N	386	192	28	P
Age (years)	42.0 (2.9)	42.0 (2.8)	42.0 (2.7)	0.969
Height (cm)	1.70 (0.92)	1.70 (0.93)	1.68 (0.72)	0.543
Weight (kg)	77.0 (16.7)	75.7 (14.9)	70.0 (13.7)	0.106
BMI (kg/m^2)	26.8 (5.3)	26.2 (4.4)	24.8 (4.1)	0.194
Ca intake (mg/d)	1234 (515)	1306 (571)	1150 (411)	0.173
Vitamin D intake (µg/d)	14.9 (13.0)	15.0 (13.6)	17.1 (15.2)	0.711
25(OH)D (nmol/L)	56.8 (19.7)	53.2 (17.9)	57.3 (20.0)	0.095
Free 25(OH)D (pmol/L)	12.3 (5.10)	11.9 (4.6)	13.4 (4.70)	0.330
PTH (ng/L)	54.9 (24.4)	55.3 (26.5)	45.8 (17.0)	0.156
DBP (mg/L)	373 (89.0)	366 (88.1)	326 (64.6)	0.038
Albumin (g/L)	43.6 (2.97)	43.8 (3.08)	44.3 (2.78)	0.365
Physical activity (min/week)	475 (391)	501 (344)	438 (266)	0.587
History of regular smoking*(%)	44	51	36	0.762

ANOVA, values are shown as means (± SD). *at least one cigarette per day for six months

5.1.3 Obese adult subjects (III)

Background characteristics of women and men in BMI groups are presented in Table 9. Obese women had lower vitamin D intake than normal-weight women, but calcium intake did not differ between the groups. Obese women were physically less active and they smoked more than normal-weight women. In obese men, vitamin D and calcium intakes were higher and they smoked more than normal-weight men. Vitamin D intake reached the current recommendations of the Nordic countries (10 μg/d) (Nordic Nutrition Recommendations 2012) in all groups.

Table 9. Characteristics of the subjects stratified by sex and BMI (III).

	Women				Men			
	Normal-weight	**Over-weight**	**Obese**	**P**	**Normal-weight**	**Over-weight**	**Obese**	
Number of subjects	186	130	68		56	72	36	P
Age (years)	41.8 (2.7)	42.1 (2.7)	42.0(2.8)	0.471	42.1 (3.1)	42.2 (3.2)	42.5 (2.8)	0.493
Height (cm)	166 (6.5)	165 (5.5)	163 (7.1)	0.001	180 (7.2)	180 (6.5)	178 (4.8)	0.092
Weight (kg)	62 (6.4)	73.7 (6.5)	89 (10)	<0.001	74.7 (7.0)	87.4 (7.2)	102.8 (9.7)	<0.001
BMI (kg/m^2)	22 (1.6)	27 (1.5)	33 (2.7)	<0.001	23.0 (1.40)	27.0 (1.36)	32.6 (2.30)	<0.001
Vitamin D intake (µg/d)*	16.2 (14)	15.1 (13)	11.1 (7)	0.019	11.3 (6.10)	16.4 (16.9)	18.4 (19.2)	0.061
Calcium intake (mg/d)*	1238 (537)	1281 (515)	1207 (489)	0.603	1121 (461)	1291 (516)	1445 (661)	0.003
Physical activity (min/week)	264 (270)	257 (203)	201 (186)	0.045	196 (178)	199 (148)	229 (353)	0.717
Holidays in sunny locations**	31%	16%	4%		9%	14%	4%	
Smoking (pack-years)	2.7 (6.2)	3.9 (6.8)	4.8 (6.7)	0.045	3.9 (9.5)	5.6 (9.5)	9.3 (11.6)	0.026
DBP (mg/L)	358 (89)	375 (92)	(102)	0.007	357 (79.4)	362 (74.2)	377 (81.6)	0.463
25(OH)D (nmol/L)	58.4 (19.5)	56.9 (21.2)	50.1 (17.6)	0.002	54.5 (17.3)	54.9 (19.2)	49.7 (18.9)	0.174
Free 25(OH)D (pmol/L)	13.1 (4.7)	12.4 (5.7)	10.0 (3.9)	<0.001	12.1 (4.5)	11.9 (4.8)	10.6 (4.7)	0.271
Bioavailable 25(OH)D (nmol/L)	5.2 (1.9)	4.90 (2.3)	3.8 (1.5)	<0.001	4.9 (1.88)	4.8 (1.79)	4.3 (1.87)	0.232
Albumin (g/L)	43.4 (2.7)	43.0 (3.0)	42.2 (2.7)	0.012	45.3 (2.86)	45.0 (3.2)	45.0 (2.41)	0.811
PTH (ng/L)	53.3 (24.6)	57.3 (26.1)	62.3 (27.7)	0.042	50.9 (20.9)	50.3 (24.4)	55.3 (22.6)	0.493
CTX (ng/mL)	0.37 (0.16)	0.33 (0.15)	0.29 (0.13)	0.001	0.54 (0.18)	0.49 (0.19)	0.39 (0.12)	<0.001
PINP (ng/mL)	36.7 (14)	35.2 (13)	32.6 (12)	0.100	43.9 (13)	40.7 (14)	37.1 (11)	0.036
Osteocalcin (ng/mL)	7.8 (2.7)	7.4 (2.7)	6.3 (2.3)	0.001	9.14 (2.4)	8.3 (2.6)	7.3 (2.0)	0.001

*Vitamin D/calcium intake from food and supplements, **Proportion of participants who spent holidays in sunny locations.
ANOVA, values are means (± SD).

5.2 Genotype and allele distributions of *GC* SNP's (I and II)

The genotype and allele distributions in Studies I and II are described in Table 10. In Study I, six *GC* diplotypes were identified: 1S/1S, 1S/2, 1F/1F, 1F/2, 1S/1F, and 2/2. Because of only a small number of subjects among some of the diplotype groups, only the data of SNP rs4588 genotypes were further analysed. The most common *GC* genotype was 1/1 and the rarest 2/2. In Study II, the same six diplotypes were detected and there were also a few individuals with undetectable genotypes. Three common haplotypes were found: GC1S, GC1F, and GC2. In Study II, the distribution of the third SNP rs705124 was 61% genotype 1, 34% genotype 2, and 5% genotype 3 (data not shown). The genotype distributions were in compliance with Hardy–Weinberg equilibrium in both studies.

Table 10. Distribution of the *GC* SNPs in Studies I and II

	Study I	**Study II**
Genotypes	%	%
*GC*1/1	68	64
*GC*1/2	26	31.5
*GC*2/2	5.6	4.5
Diplotypes		
*GC*S/1S	-	47
*GC*1S/2	-	25
*GC*1F/1F	-	2
*GC*1F/2	-	6
*GC*1F/1S	-	16
*GC*2/2	-	4
Haplotypes		
GC1S	-	88
GC1F	-	3.7
GC2	-	8.5

5.3. 25(OH)D and PTH concentrations among *GC* genotypes (I, II)

5.3.1 Children and adolescents (I)

After adjustment with vitamin D intake, PTH, study group, and month of sampling, 25(OH)D concentrations differed significantly among the genotypes being highest in *GC*1/1 and lowest in *GC*2/2 (P=0.001, ANCOVA) (Figure 5A). In pairwise comparison, only the difference between genotypes *GC*1/1 and *GC*1/2 was significant (P=0.003). There was thus a negative linear trend among the three genotypes (P=0.025). When adjusted with Ca intake, 25(OH)D and month of sampling, PTH concentration was significantly higher in *GC* 1/1 than in the other genotypes (Figure 5 B). Similarly, in individuals with the genotype *GC*1/2 the 25(OH)D concentration was intermediate and they also had intermediate PTH concentration, and in individuals with the *GC*2/2 genotype, who had the lowest 25(OH)D concentration, the lowest PTH concentration (P=0.028, linear trend P=0.012) was seen.

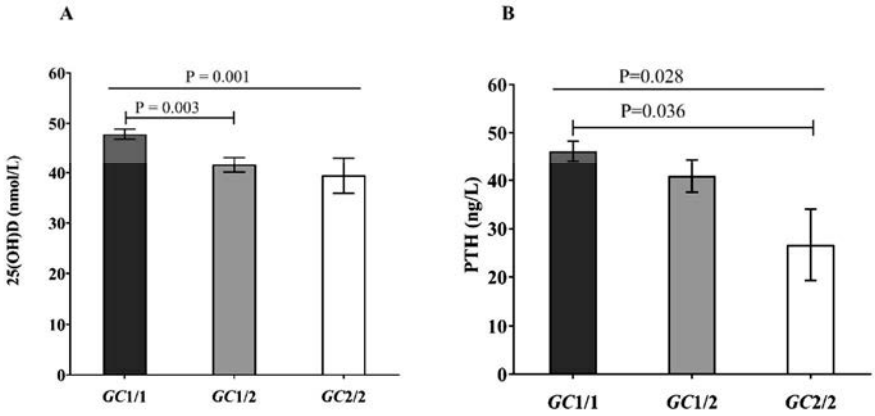

Figure 5 A-B Association of GC genotypes with 25(OH)D (A) and PTH (B) concentrations in children and adolescents in Study I. Results are shown as estimated means ± SE, ANCOVA.

5.3.2 Adults (II)

25(OH)D concentrations differed among the SNP rs4588 genotypes (P=0.031, ANCOVA) and also between the six diplotypes (P=0.033, ANCOVA). PTH, vitamin D intake, sunlight exposure, BMI and gender were used as covariates (Figure 5 C). Similarly, PTH concentrations differed between the diplotypes (P=0.040), when adjusted for 25(OH)D, calcium intake and sex (Figure 5 D). Among the haplotypes there was no significant difference (P=0.078). However, a linear trend was emerged (P=0.044, data not shown). Among the SNP rs705124 genotypes, a difference between PTH concentrations was discovered (P=0.016, ANCOVA, data not shown).

5.4 Association of *GC* diplo/haplotype with DBP, total, free and bioavailable 25(OH)D (II)

DBP concentrations differed among the diplotype and haplotype groups (P = 0.039 and P=0.039, respectively, ANCOVA), one of the lowest concentration being in subjects with the *GC*2/2 genotype (Figure 5 E).There was no difference in free and bioavailable 25(OH)D concentrations among the *GC* diplo-/haplotypes. However, in genotype-adjusted concentrations the highest concentration of free 25(OH)D was found in diplotype 2/2 (P<0.001, ANCOVA) (Figure 5 F), and haplotype *GC*2 (P<0.001, data not shown). Also bioavailable 25(OH)D concentrations differed significantly among the genotypes (P<0.001) and similarly, highest concentration was found in genotype *GC*2/2. (Figure 5 G).

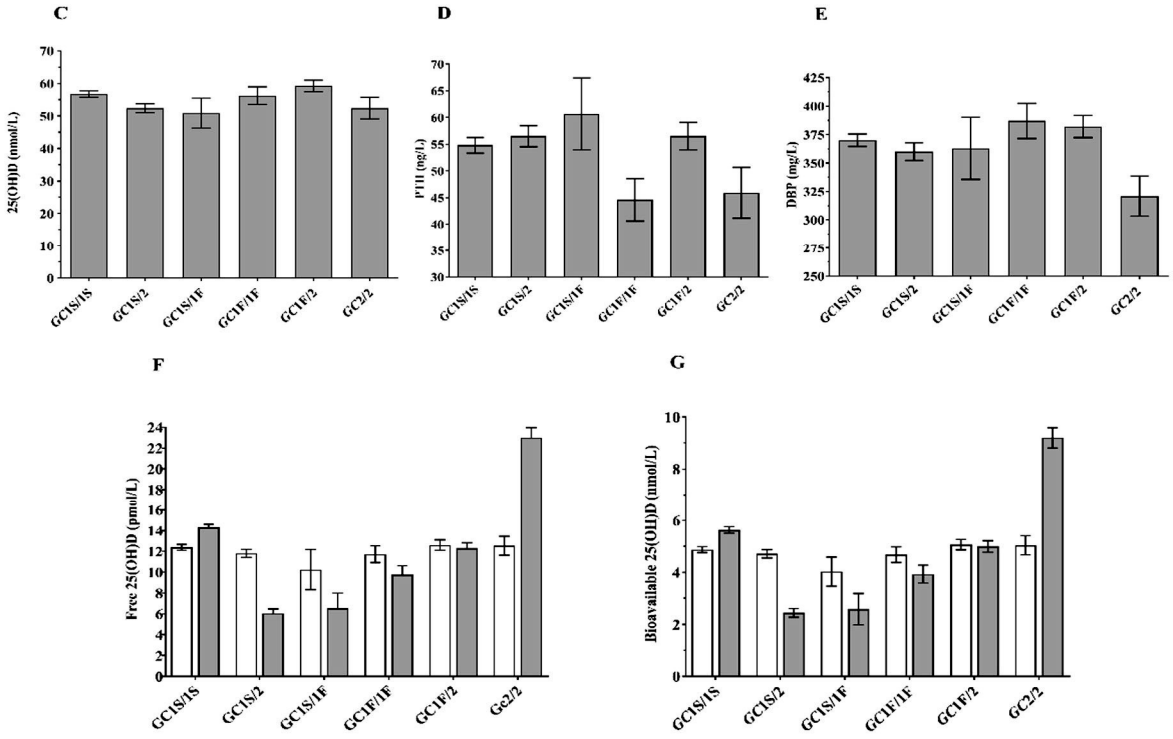

Figure 5 C-G Association of *GC* diplotypes with 25(OH)D (C), PTH (D), DBP (E), free 25(OH)D (F) and bioavailable 25(OH)D (G) concentrations in Study II. White bars are unadjusted concentrations and grey bars are adjusted with genotype specific binding constants (F and G). Results are shown as estimated means ± SE, ANCOVA

5.5 Total, free and bioavailable 25(OH)D concentrations between BMI groups (III)

Total, free, and bioavailable 25(OH)D correlated inversely with BMI in women (r=-0,20; -0,21; -0,23, respectively, P<0.001) and in both genders analysed together (r=-0.15; -0.19; -0.19; respectively, P<0.001), but not in men. In women, 25(OH)D concentrations did not differ among the BMI groups (Figure 6 A). In men, 25(OH)D was lower in obese men than in their normal-weight peers (P=0.024) but no differences emerged between normal-weight and overweight groups or overweight and obese groups. Similarly, when both genders were analysed together, obese subjects had lower 25(OH)D than normal-weight subjects (P=0.003). There was also a significant difference between overweight and obese groups (P=0.048). Free and bioavailable 25(OH)D concentrations were lower in obese women (P<0.008 and <0.003, respectively) (Figures 6 B and 6 C). Also in men, free and bioavailable 25(OH)D were lower in obese subjects (P=0.044 and 0.032, respectively). When both genders were analysed together, a significant difference remained (P<0.001). There was also a significant difference between overweight and obese subjects in free and bioavailable 25(OH)D. No differences was seen between normal-weight and overweight group or overweight and obese groups in women and men analysed separately.

5.6 PTH and DBP concentrations between BMI groups (III)

Total and free 25(OH)D correlated negatively with PTH in both men (r= -0.179, P= 0.013 and r= -0.159 P=0.042, respectively) and women (r= -0.232, P<0.001, and r= -0.211, P<0.001, respectively). Bioavailable 25(OH)D correlated negatively with PTH in women (r=-0.207; P <0.001), but in men the correlation did not reach significance. DBP correlated positively with BMI when men and women were analysed together (r=0.363, P=0.025). In women, DBP and PTH correlated positively with BMI (r=0.129, p=0.016; r=0.181, P<0.001, respectively). In women, PTH was higher in obese subjects than in normal-weight subjects (P=0.045) (Figure 6 D). In men, the difference was not significant. When both sexes were analysed together, the difference was significant (P=0.047). Obese women had higher DBP concentration than normal-weight women (P=0.008) (Figure 6 E). In males, DBP concentration did not differ among BMI groups. When both sexes were analysed together, DBP was higher in obese subjects than in normal-weight subjects (P=0.004). There was no difference in PTH or DBP concentrations between normal-weight and overweight subjects or overweight and obese subjects.

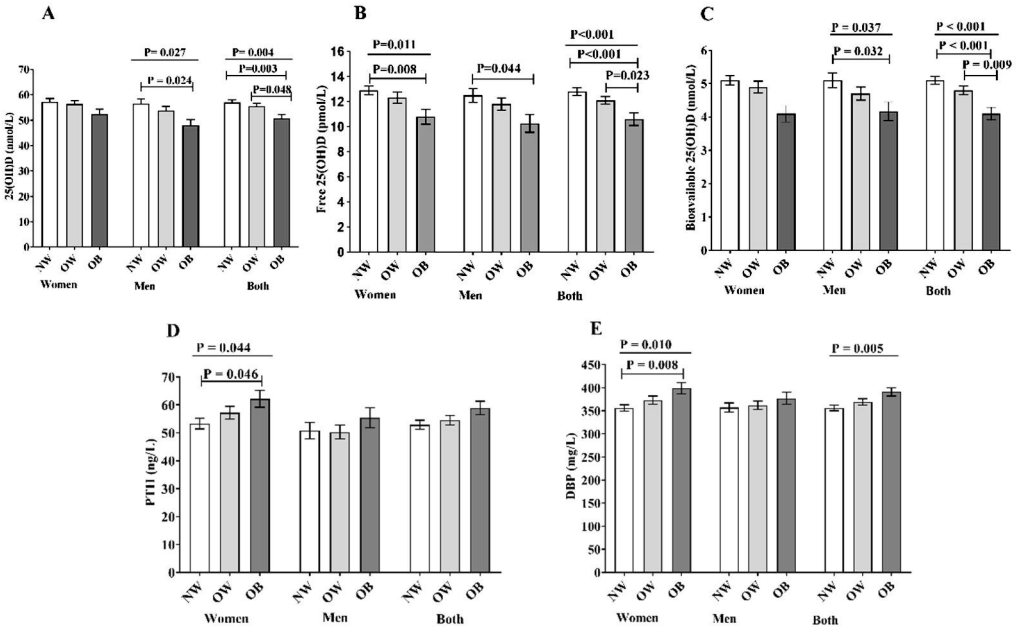

Figure 6 Differences in total (A), free (B) and bioavailable 25(OH)D (C), PTH (D) and DBP (E) concentrations between normal-weight (NW), overweight (OW), and obese (OB) women, men, and both sexes combined. Results are shown as mean (±SE). The values were adjusted for vitamin D intake, age, and holidays in sunny locations (A,B,C), calcium intake (D) or use of hormonal contraceptives (E). ANCOVA, Bonferroni pairwise comparisons between normal-weight and obese subjects.

5.7 Associations of *GC* genotypes with skeletal outcomes (I)

The unadjusted values of bone parameters measured with DXA among genotypes GC 1/1 and the combined genotypes GC 1/2 and 2/2 in children and adolescents are presented in Table 11 (ANOVA). There was no differences in bone traits among the genotypes in girls and boys. However, in regression analysis, GC genotype together with weight, age and pubertal stage were significant determinants of LS BMC in both boys and girls, as well as height in girls. Also when girls and boys were analysed together, GC genotype remained a significant determinant of LS BMC. In girls, GC genotype together with height, 25(OH)D concentration, pubertal stage, and age were significant determinants of total hip BMC. In addition, weight, 25(OH)D concentration, GC genotype and PTH in girls determined SSI. The results of linear regression analysis on determinants of bone characteristics in groups defined by sex are presented in Tables 12 and 13.

When the bone variables were transformed into Z-scores, GC genotype was a significant determinant of LS BMC Z-score in girls and weight, 25(OH)D concentration, GC genotype and physical activity were significant determinants of SSI Z-score when genders were combine

Table 11. Bone variables for genotype *GC* 1/1 and genotypes GC 1/2 and 2/2 combined, in girls and boys measured with DXA

	Girls			**Boys**		
Genotype	***GC 1/1***	***GC 1/2* and *2/2***		***GC 1/1***	***GC 1/2* and *2/2***	
N	105	55	**P**	53	18	**P**
LS area (cm^2)	46.9 (8.8)	46.9 (9.3)	0.959	48.6 (11.6)	45.6 (11.3)	0.344
LS BMC (g)	38.2 (13.3)	38.6 (14.9)	0.876	39.2 (17.4)	33.7 (15.7)	0.239
LS BMD (g/cm^2)	0.79 (0.15)	0.80 (0.16)	0.801	0.77 (0.17)	0.71 (0.15)	0.158
LS BMD Z	-0.003 (0.98)	-0.05 (1.01)	0.762	0.39 (0.92)	-0.20 (67)	0.014
Total hip area, (cm^2)	28.4 (4.1)	28.1 (4.5)	0.698	30.7 (7.78)	29.2 (8.4)	0.506
Total hip BMC (g)	23.1 (5.6)	23.1 (6.7)	0.947	27.1 (10.5)	24.7 (10.4)	0.41
Total hip BMD (g/cm^2)	0.81 (0.11)	0.810 (0.14)	0.894	0.86 (0.14)	0.82 (0.13)	0.355
Total hip BMD Z	0.23 (0.9)	0.191 (0.96)	0.809	0.21 (0.87)	0.06 (0.68)	0.510
N	75	35		53	18	
WB a (cm^2)	2147 (320)	2143 (341)	0.951	2130 (447)	2060 (425)	0.554
WB BMC (g)	1791 (421)	1831 (478)	0.652	1820 (614)	1648 (527)	0.290
WB BMD (g/cm^2)	0.82 (0.09)	0.84 (0.11)	0.361	0.84 (0.123)	0.78 (0.10)	0.117
WB BMD Z	-0.020 (0.79)	0.006 (0.8)	0.881	0.23 (0.87)	-0.26 (0.62)	0.029

Values are presented as mean (± SD). ANOVA. LS=lumbar spine, WB=whole body, BMC=bone mineral content, BMD=bone mineral density, Z=age- and sex- adjusted value Z-score).

Table 12. Linear regression analysis for determinants of bone characteristics in groups defined by sex (I).

	LSBMC			**Total hip BMC**			**WB BMC**		
Girls (N=159)	R^2	ß	P	R^2	ß	P	R^2	ß	P
Regression model	**0.873**		**≤0.001**	**0.824**		**≤0.001**	**0.748**		≤0.001
Height (cm)	**0.003**	0.124	**0.021**	**0.131**	0.248	**≤0.001**	-	0.006	0.934
Weight (kg)	**0.015**	0.111	**0.016**	—	0.044	0.417	**0.578**	**0.475**	**≤0.001**
Pubertal stage	**0.005**	-0.082	**0.014**	**0.011**	-0.126	**0.001**	**0.067**	**0.201**	**≤0.001**
25(OH)D (nmol/L)	**0.629**	0.522	**≤0.001**	**0.655**	0.565	**≤0.001**	**0.005**	**-0.116**	**0.031**
GC genotype*	**0.003**	0.065	**0.030**	**0.017**	0.105	**0.003**	—	-0.062	0.144
Physical activity score	—	-0.033	0.255	—	-0.024	0.480	—	0.020	0.627
Age	**0.218**	0.357	**≤0.001**	**0.010**	0.179	**0.002**	**0.098**	**0.496**	**≤0.001**
Boys (N=73)									
Regression model	**0.812**		**≤0.001**	**0.824**		**≤0.001**	**0.873**		**≤0.001**
Height (cm)	—	0.001	**0.997**	**0.045**	0.228	**≤0.001**	—	0.073	0.563
Weight (kg)	**0.030**	0.269	**0.017**	**0.014**	0.228	**0.032**	**0.061**	**0.382**	**≤0.001**
Pubertal stage	**0.031**	0.297	**0.003**	**0.015**	0.249	**0.008**	**0.019**	**0.188**	**0.020**
25(OH)D (nmol/L)	—	0.007	0.894	—	0.069	0.191	—	0.084	0.066
GC genotype*	**0.010**	-0.019	**0.049**	—	-0.073	0.161	**0.008**	**-0.093**	**0.041**
Physical activity score	—	-0.015	**0.807**	—	-0.742	0.461	—	-0.003	0.954
Age	**0.741**	0.416	**0.002**	**0.750**	0.287	**0.019**	**0.785**	**0.394**	**≤0.001**

R^2 values are adjusted and ß2values are standardized. BMC=bone mineral content, LS= lumbar spine, WB= whole body. * GC genotype is used as a dummy variable.

— =variable not included in the stepwise regression model. Determinants with bold R^2 and P-value are included in the model.

Table 13. Linear regression analysis for determinants of lumbar spine, total hip and whole body BMD and SSI Z-score in groups defined by sex (I).

	LS BMD Z			**Total hip BMD Z**			**WB Z**			**SSI Z**		
Girls (N=159)	R^2	ß	P	R^2	ß	P	R^2	ß	P	R^2	ß	P
Regression model	**0.260**		**≤0.001**	0.237		≤0.001	**0.143**		**0.003**	**0.265**		**≤0.001**
Height (cm)	**0.097**	-0.503	**≤0.001**	0.232	-0.654	≤0.001	**0.064**	-0.506	**0.002**		-0.668	0.060
Weight (kg)	**0.163**	0.797	**≤0.001**	0.005	0.739	≤0.001	—	0.245	0.110	**0.104**	0.525	**0.003**
Pubertal stage	—	0.080	0.276		0.066	0.413	—	0.093	0.407		0.241	0.060
25(OH)D (nmol/L)	—	0.025	0.769		-0.906	0.339	**0.079**	0.451	**≤0.001**	**0.051**	-0.529	**0.010**
GC genotype*	—	-0.077	0.276		-0.050	0.482	—	-0.046	0.645	**0.028**	-0.272	**0.011**
Physical activity score	—	0.037	0.532		0.073	0.303	—	0.010	0.921	—	0.424	0.086
PTH	—									**0.082**	0.603	**0.005**
Boys (N=73)				N=56				N=73		N=42		
Regression model	**0.162**		**0.018**	**0.090**		0.015	**0.195**		**0.015**	**0.082**		**0.187**
Height (cm)	**0.039**	-0.540	**0.042**		-0.478	0.198	—	-0.023	0.940	—	-0.728	0.124
Weight (kg)	**0.056**	0.659	**0.006**	**0.090**	4.474	0.006	—	-0.113	0.663	—	0.654	0.097
Pubertal stage		0.001	0.991		-0.191	0.477	—	0.102	0.616	**0.067**	0.536	**0.043**
25(OH)D (nmol/L)	—	-0.017	0.885		0.081	0.564	—	0.219	0.067	-	-0.008	0.962
GC genotype*	**0.067**	-0.243	**0.039**		-0.138	0.335	**0.049**	-0.252	**0.053**	-	0.004	0.982
Physical activity score	—	0.001	0.991		0.091	0.584	**0.146**	0.356	**0.023**	**0.015**	0.431	**0.018**
PTH	—									—	-0.033	0.833

R^2 values are adjusted and ß2 values are standardized. PTH was included in the SSI model. BMD=bone mineral density, LS= lumbar spine, WB= =whole body, SSI= strength strain index* GC genotype is used as a dummy variable. Z= Z-score

— =variable not included in the stepwise regression model. Determinants with bold R^2 and P-value are included in the model.

5.8 Obesity and bone (III)

5.8.1 Differences in bone traits between BMI groups

Mean ±SD bone traits and comparison between normal-weight (NW), overweight (OW) and obese (OB) subjects are shown in Table 14. Trabecular density in obese women was 6.3% higher in distal radius and 6.6% higher in the distal tibia than the corresponding figures in normal-weight women ($P=0.004$ and $P<0.001$, respectively). In addition, CSI was 12.5% higher in obese women than in normal-weight women ($P=0.022$). Cortical density was 1.7% and 0.9% lower in obese women in the shaft sites of the radius and tibia, respectively ($P<0.001$; $P=0.008$, respectively) (ANOVA). In obese men, trabecular density was 3.9% higher in the distal radius and 12% higher in the distal tibia than the corresponding figures in normal-weight men ($P=0.012$ and $P<0.001$, respectively) (ANOVA). Distal tibia CSI was 11% higher in overweight and obese group compared to normal-weight and tibial shaft CSI 1% higher in obese and 2 % higher in overweight group ($P=0.029$; $P=0.019$, respectively). Tibial shaft cortical density was 1% lower in obese men than in normal-weight men ($P<0.001$).

5.8.2 Differences in bone turnover markers between the BMI groups

Mean values of bone turnover markers and comparison between normal-weight, overweight and obese subjects are presented in Table 14. CTX and PINP concentrations were lower in obese women than in normal-weight women after adjusting with age. Also PINP and osteocalcin were lower in obese women. In men, CTX and osteocalcin concentrations were lower in obese subjects than in normal-weight subjects.

Table 14. pQCT-bone characteristics of subjects stratified by sex and BMI (III).

	Women				Men			
	NW	**OW**	**OB**	**P**	**NW**	**OW**	**OB**	**P**
CTX (ng/mL)	0.37 (0.16)	0.33 (0.15)	0.29 (0.13)	0.001	0.54 (0.18)	0.49 (0.19)	0.39 (0.12)	<0.001
PINP (ng/mL)	36.7 (14)	35.2 (13)	32.6 (12)	0.1	43.9 (13)	40.7 (14)	37.1 (11)	0.036
Osteocalcin (ng/mL)	7.8 (2.7)	7.4 (2.7)	6.3 (2.3)	0.001	9.14 (2.4)	8.3 (2.6)	7.3 (2.0)	0.001
pQCT traits	N=168	N=120	N= 62		N=65	N=90	N=42	
Distal radius TraD (mg/cm^3)	192 (29.0)	198 (26.5)	206 (28)	0.004	219 (28)	232 (26)	228 (26)	0.012
Distal radius CSI	0.25 (0.06)	0.25 (0.05)	0.26 (0.06)	0.555	0.28 (0.06)	0.27 (0.05)	0.27 (0.06)	0.853
Radial shaft CorD (mg/cm^3)	1148 (33)	1133 (39)	1128 (41)	<0.001	1124 (49)	1117 (39)	1109 (34)	0.187
Radial shaft CSI	0.86 (0.07)	0.87 (0.04)	0.87 (0.55)	0.464	0.85 (0.05)	0.85 (0.05)	0.86 (0.04)	0.704
Distal tibia TraD (mg/cm^3)	209 (30)	219 (24)	223 (25)	<0.001	225 (27)	243 (28)	237 (28)	<0.001
Distal tibia CSI	0.21 (0.06)	0.22 (0.04)	0.24 (0.05)	0.022	0.24 (0.05)	0.27 (0.07)	0.27 (0.06)	0.029
Tibial shaft CorD (mg/cm)^3	1110 (29)	1102 (26)	1100 (28)	0.008	1106 (25)	1088 (26)	1094 (28)	<0.001
Tibial shaft CSI	0.80 (0.05)	0.80 (0.05)	0.81 (0.05)	0.230	0.80 (0.05)	0.82 (0.04)	0.81 (0.04)	0.019

ANOVA, NW=normal-weight, OW=overweight, OB=obese, CorD=cortical density, TraD= trabecular density, CSI= cortical strength index. Means (±SD)

5.8.3 Associations of total, free and bioavailable 25(OH)D with bone traits in BMI groups

Standardized β-coefficients and R^2 -values in backward linear regression models for determinants of pQCT-bone traits in obese women are presented in Table 15. 25(OH)D, free and bioavailable 25(OH)D were included as independent variables in regression models 1, 2 and 3, respectively. In model 1, there was a negative association of 25(OH)D with distal radius TraD and tibial shaft CSI. Furthermore, in model 2, free 25(OH)D was a negative determinant of distal radius CSI and tibial shaft CSI and radial shaft CoD. In model 3, bioavailable 25(OH)D was a negative determinant of distal radius CSI, and tibial shaft CSI and radial shaft CorD. In normal-weight and overweight women, no associations were found between bone traits and 25(OH)D, free, or bioavailable 25(OH)D concentrations. In normal-weight and obese men, no associations were found between 25(OH)D concentrations and bone traits. In overweight men, a positive association emerged between total 25(OH)D and distal radius CSI (data not shown).

Table 15. Standardized β-coefficients and R^2 -values in backward linear regression models for determinants of pQCT-bone traits in obese women (Study III). Other determinants in the models were age, physical activity, and smoking.

	Model 1: 25(OH)D			**Model 2: Free 25(OH)D**			**Model 3: Bioavailable 25(OH)D**		
Obese women N= 62	Adjusted R^2	β	P	Adjusted R^2	β	P	Adjusted R^2	β	P
Distal radius TraD (mg/cm^3)	0.089	-0.346	0.009	0.027	-0.214	0.100	0.016	-0.189	0.147
Distal radius CSI	0.028	-0.152	0.243	0.070	-0.255	0.049	0.072	-0.260	0.045
Radial shaft CorD (mg/cm^3)	0.016	-0.180	0.162	0.050	-0.255	0.045	0.059	-0.273	0.032
Tibial shaft CSI	0.146	-0.372	0.004	0.113	-0.317	0.012	0.107	-0.308	0.015

R^2 values are adjusted and ß2 -values are standardized. CorD= cortical density, TraD=trabecular density, CSI=cortical strength index.

6 DISCUSSION

6.1 Main findings

In both children and adolescent in Study I as well as in middle-aged men and women in Study II, an association of 25(OH)D concentration with *GC* gene polymorphisms was found. In addition, *GC* genotype was associated with DBP concentrations and with free and bioavailable 25(OH)D concentrations in Study II. *GC* genotype was associated with BMD and bone strength measured with pQCT/DXA in Study I. In obese middle-aged subjects, lower total, free and bioavailable 25(OH)D and higher DBP and PTH concentrations than in normal-weight subjects were presented (Study III). Total, free and bioavailable 25(OH)D concentrations were associated with bone traits in obese subjects and markers of bone metabolism differed between obese and normal- weight subjects.

6.2. *GC* genotype distribution

Three *GC* genotypes: 1/1, 1/2 and 2/2 and six diplotypes: GC1S/1S, GC1S/2, GC1F/1, GC1F/2, GC1F/1S, and GC2/2 were detected in Studies I and II. Unfortunately in Study I, the number of subjects was too low to conduct statistical analyses of diplotypes. The genotype distribution in children and adolescents in Study I and in adults in Study II was similar. Three different haplotypes were detected in Study II, corresponding to the three GC protein isoforms GC1S, GC1F and GC2. The genotype distribution in Studies I and II resembles the genotype distribution of GC gene reported in Finnish genome bank databases (Sequencing Initiative Suomi=SISu) (total 10 481 samples). The genotype distribution in SISu is *GC* 1/1=63 % (N=6665), *GC*1/2=32 % (N=3406), *GC* 2/2 =3,9 % (N=410).

The frequency of different DBP isotypes varies by ancestry; *GC* 1F/1F is most common in West Africans and African Americans and least common in Caucasians (Kamboh & Ferrel 1986). Finns have been shown to be genetically distant from Central Europeans

or even from the inhabitants of other Nordic countries (Salmela *et al*. 2011). For example in a study conducted in Danes, the distribution of the GC phenotypes GC1S/1S, GC1S/1F, Gc1F/1F, GC1S/2, GC1F/2 and GC2/2 was 32%, 19%, 2.9%, 33.4%, 5.2% and 7%, respectively (Lauridsen *et al.* 2005). For our knowledge, there is no published data on the GC diplotype distribution in Finns.

6.3 Total, free and bioavailable 25(OH)D, PTH and DBP in *GC* genotypes

DBP variants have been shown to have affinity differences to 25(OH)D and 1,25(OH)D due to the differences in protein configuration. GC1F has the highest affinity and GC2 the lowest (Arnaud & Constans 1993). The same hierarchy has been found in serum DBP concentrations (Lauridsen *et al.* 2001). In children and adolescents of Study I and in adults of Study II, serum 25(OH)D concentrations differed considerably among the *GC* genotypes, the concentration being highest in genotype *GC* 1/1 and lowest in *GC* 2/2. In diplotypes, 25(OH)D concentration was lowest in 1S/1F and highest in 1F/2. The difference between 25(OH)D concentrations was significant between diplotypes 1S/2 and 1F/2. The study by Lauridsen *et al.* (2001) was one of the first to report the common SNP's rs4588 and rs7041 to be associated with 25(OH)D concentrations, and several studies, including Studies I and II, have since confirmed this finding (Lauridsen *et al*. 2005, Sinotte 2009, Gozdzik *et al*. 2011, Carpenter *et al.* 2013, Nissen *et al.* 2014, Powe *et al*. 2014, Petersen *et al*. 2017). The findings have been similar across studies, with the C allele (ancestral allele) of rs4588 associated with higher 25(OH)D concentrations. Similarly in SNP rs7041, the findings have been consistent with lower 25(OH)D with the T allele (ancestral allele).

Nissen *et al*. (2014) found that six SNPs in the *GC* gene (rs4588, rs16846876, rs2282679, rs12512631, rs17467825, and rs842999) were associated with 25(OH)D concentrations in Danes. Recent longitudinal study including 642 healthy Danish children, investigated associations between vitamin D-dependent SNPs and 25(OH)D concentrations (Petersen *et al*. 2017). They found minor alleles of *GC* rs4588 and rs7041 to be associated with lower serum 25(OH)D concentrations across the seasons. In a study by Powe *et al.* (2014), genetic polymorphism in the GC gene explained almost 10% of the variation in 25(OH)D concentrations.

In Studies II and III, one aim was to test whether the unbound fractions of vitamin D were more biologically active than the bound 25(OH)D. The concentrations of free and bioavailable 25(OH)D were calculated with a previously published formula (Bikle *et al.* 1986, Powe *et al.* 2014). Furthermore, an adjustment was made for the free and bioavailable 25(OH)D concentrations with haplotype- and diplotype-specific binding constants (Arnaud & Constans 1993, Johnsen *et al.* 2014) in Study II. Free and bioavailable concentrations differed significantly among the *GC* haplotypes and diplotypes after the adjustment. Also in a Norwegian study, calculated free and bioavailable concentrations changed considerably, when they were adjusted for DBP binding constants (Johnsen *et al*. 2014).

Due to the inverse association between 25(OH)D and PTH found in numerous studies, PTH concentration has been suggested for use as a reference for optimal vitamin D status (Sai *et al.* 2011). As expected, there was an inverse correlation between 25(OH)D and PTH in both children and adolescents in Study I and in adults in Study II. In contrast to general associations, in Study I, both 25(OH)D and PTH were lowest in the *GC*2/2 genotype.

In Study II, the similar trend as in Study I was seen: *GC*2/2, with the second lowest 25(OH)D concentrations among diplotypes, had the lowest PTH concentrations. Interestingly, the free and bioavailable 25(OH)D concentrations were high in these groups as well, which could be one explanation for the low PTH concentrations.

It has been shown that vitamin D prohormones can suppress PTH secretion from the parathyroid glands through the VDR receptor (Ritter *et al*. 2011). Because the amount of free 25(OH)D was higher in genotype *GC*2/2, there could be more free forms of 25(OH)D which could suppress PTH secretion from the parathyroid glands. Carpenter *et al.* 2013 observed that *GC* genotype did not have an influence on the relationship between 25(OH)D and PTH. However, they did not investigate the association of PTH with GC genotypes.

DBP concentrations varied significantly among the *GC* genotypes as well as among the haplotypes in Study II, the lowest DBP concentration being in genotype *GC*2/2 and the highest in *GC*1/1. In diplotypes, the highest concentration was in *GC*1F/1F and the lowest in *GC*2/2. However, the only significant difference was between *GC*1F/2 and *GC*2/2. Several studies have found variation in DBP concentrations among the common GC genotypes (Lauridsen *et al.* 2001, Nissen *et al.* 2014, Wilson *et al.* 2015). In a study of African American and Caucasian American subjects, DBP concentration was highest in *GC*1S homozygotes, intermediate in *GC*2, and lowest in *GC*1F (Wilson *et al.* 2015).

According to a Danish study, *GC*2 homozygotes had lower DBP concentrations than the other genotypes, which is in line with the results of Study II (Lauridsen *et al.* 2001).

Vitamin D intake and 25(OH)D concentrations

In Study I, 67 % of the children and adolescents had 25(OH)D concentration rather low; below 50 nmol/L. Vitamin D intake from food and supplements was quite low also, especially in girls (< 10 µ/day). The explanation for this is probably the fact that the study data was collected between the years 2001-2002 (Optiford) and 2007-2008, when the fortification of foodstuffs with vitamin D was not yet at the same level as at the present. The recommendations for vitamin D fortification were doubled in 2010 for milk and spreads. Also the recommendations for total vitamin D intake were increased from 7.5 to 10 µg/day in 2014. In adults (Study II and III), mean serum 25(OH)D concentration was 56 nmol/L. The mean vitamin D intake from food and supplements was 16 µg/day and there was a large variation among the subjects. The PHOMI-study was conducted in spring 2010, when the fortification of fluid milk products and spreads was already more common. The higher vitamin D intakes and 25(OH)D concentrations in the adults are most likely due to fortification and increased use of supplements.

6.4 Relationship between *GC* genotype and bone parameters in children and adolescents

Several studies have examined *GC* gene polymorphism and bone health. Most of them have concentrated on the common SNPs rs4588 and rs7041 (Lauridsen *et al.* 2004, Al-Oanzi *et al.* 2006, Fang *et al.* 2009, Xu *et al.* 2010), whereas some have investigated other polymorphisms in the *GC* gene (Papiha *et al.* 1999, Ezura *et al.* 2003). The majority of studies have focused on adults. Study I is novel in evaluating the association between *GC* genotype and bone health in children and adolescents. When the mean bone traits between the genotypes in girls and boys were compared with ANOVA and ANCOVA, the only difference was found in total hip BMC in boys. The group with the

combined GC1/1 and GC2/2 genotype had slightly lower BMC in total hip. Also in boys, GC genotype was a negative determinant of lumbar spine BMD and whole body BMD. In girls, genotype was a negative determinant of the bone strength and strain index, SSI. Low SSI, volumetric BMD, cortical area and areal BMD can contribute to increasing fracture risk in children (Kalkwarf *et al.* 2011). Also other studies have reported association of GC polymorphism with bone strength indices and fracture risk. In a study on Japanese postmenopausal women, SNPs within the *GC* gene were associated with BMD of the radius measured with DXA (Ezura *et al.* 2003). The authors concluded that several SNP's in the GC gene could act together and increase the osteoporosis risk. In a study by Lauridsen *et al.* (2004), a significant difference in BMD in postmenopausal women among *GC* phenotypes was found. DBP polymorphism has been shown to be associated with fracture risk in men from United Kingdom (Al-Oanzi *et al.* 2006). Fang *et al*. (2009) reported an association between haplotype *GC*1S and fracture risk. The risk was stronger when dietary Ca intake was low. In study I, Ca intake was quite high, but there was still an association between SSI and genotype. Xu *et al.* (2010) reported that there was an association between compression strength index and GC polymorphism in Caucasian men.

The mechanism how GC genotype affects bone metabolism is not clear. Lauridsen *et al*. 2004 suggested that the association they found between GC genotype and fracture risk could arise from GC-MAF formation. Because DBP concentration differs between DBP genotypes, there would be more substrate for GC-MAF formation in certain genotypes. Additionally, they found that osteoclast formation rate was higher. Another reason could be different glycosylation of DBP isoforms because the formation of GC-MAF requires removal of the glucose residuals (Lauridsen 2004). Unfortunately DBP concentration were not measured in Study I. It is possible though that one reason for the negative association of GC polymorphism with bone traits found in Study I could be due to a difference in DBP concentrations among different genotypes leading to differences in GC-MAF formation and osteoclast activation (Fang *et al*. 2009).

6.5 Vitamin D metabolism and bone in obese subjects

25(OH)D concentrations are lower in obese people and have been shown to correlate inversely with BMI (Bell *et al.* 1985, Konradsen *et al.* 2008). Reasons for lower 25(OH)D could be larger volume of fat tissue as well as other tissues i.e. blood and muscles, leading to less vitamin D for 25(OH)D synthesis in the liver (Liel *et al.* 1988, Wortsman *et al.* 2000, Drincic *et al.* 2012). Studies have revealed that the rise of 25(OH)D in the circulation after UV exposure is weaker in the obese, and therefore, these subjects may need a higher vitamin D dosage than normal-weight subjects to reach the same concentration of 25(OH)D in blood (Wortsman *et al.* 2000, Drincic *et al.* 2012).

In Study III, 25(OH)D concentrations differed significantly among the BMI groups. The association was found in men and in the total group (men and women together). Also in women there was a similar trend in Study III. The lowest 25(OH)D concentrations were found in obese subjects. Although it is well established that obese individuals have lower 25(OH)D, few studies have examined whether there are also differences in free and bioavailable 25(OH)D. Furthermore, the results of the studies have been contradictory partly because the methods have varied from estimated values (Karlsson *et al.* 2014) to direct measurement (Holmlund-Suila *et al.* 2016, Walsh *et al.* 2016) and are therefore not completely comparable. A Swedish study compared obese and normal-weight women and discovered that obese women had lower total and calculated free 25(OH)D than in normal-weight women (Karlsson *et al.* 2014). Walsh *et al.* (2016) measured free and bioavailable 25(OH)D concentrations directly in obese women and men of various age groups in United Kingdom. In their study, 25(OH)D was lower in the obese and overweight groups than in normal-weight groups only in the fall and spring. However, there was a negative correlation with whole-body fat mass in all seasons. In Study III, 25(OH)D was lower among obese subjects only in men or when men and women were analysed together. Study III was conducted in winter, when the cutaneous synthesis of vitamin D is marginal in Finland. Walsh and colleagues (2016) suggested that the stronger difference between obese and normal-weight subjects in the fall and spring could be explained by the attenuated rise of 25(OH)D in the obese. Consequently, the lack of vitamin D measurements from summer months in Study III may have narrowed the range of 25(OH)D data and reduced the strength of the observed associations.

In study III we discovered that obese subjects had higher DBP concentrations than normal-weight subjects. Taes *et al.* (2006) found that DBP correlated positively with BMI and leptin concentrations. They also observed differences in fasting glucose

concentrations between DBP phenotypes and suggested that the relation DBP-fat mass could have influenced glucose handling of these subjects. In vitamin D deficiency, DBP could stabilize vitamin D concentrations with positive effects on glucose handling. Karlsson *et al.* (2014) compared 22 to 45-year-old obese and normal-weight women and found that obese individuals had higher DBP concentrations. They suggested that because oestrogens are known to increase DBP concentrations, higher concentrations of free oestrogen in obesity might have an effect on hepatic DBP production. They also suggested that IL-6 could be behind the higher DBP concentration in obese subjects, since *in vitro* IL-6 has been shown to increase hepatic DBP production (Guha *et al.* 1995) and IL-6 is elevated in obesity (Bastard 2000). Walsh *et al.* (2016) found no difference in DBP concentrations between obese and normal-weight subjects but DBP concentrations correlated inversely with BMI. Also in a Finnish study, DBP concentrations did not differ between obese and normal-weight young adults (Holmlund-Suila *et al.* 2016). Oral oestrogens and pregnancy are known to increase DBP concentrations in the circulation. Higher concentrations of free oestrogen in obesity could possibly have an effect on hepatic DBP production and be one explanation for the higher DBP concentration in obese subjects (Karlsson *et al.* 2014). Some studies have suggested that insulin might suppress the production of DBP. In these studies, DBP was lower in obese compared to normal- weight subjects and there was an inverse relationship between insulin levels and DBP, even when corrected for adiposity (Ashraf *et al.* 2014, Miraglia del Giudice *et al.* 2015). Unfortunately, in Study III we were not able to determine insulin resistance of the subjects. Miraglia del Giudice *et al.* (2015) found that obese insulin resistant children had 25(OH)D concentrations similar to those of normal-weight children due to reduced concentration of DBP.

Also DBP genotype has been demonstrated to associate with obesity although the biological mechanism for this remains obscure (Jiang *et al.* 2007, Almesri *et al.* 2016). In general, DBP genotype can influence the measured DBP concentrations as well as DBP's ability to bind 25(OH)D. In Study II, we found that GC genotype was associated with DBP, PTH, and vitamin D concentrations. Due to rather small group sizes in Study III, it was not possible to examine the role of GC genotype in vitamin D metabolism of obese subjects.

As expected, an inverse association between 25(OH)D and PTH was found in Study III. Furthermore, PTH concentrations differed between the BMI groups, but only in women or when the sexes were combined. Contrary to the results of Study III, Walsh *et al.* (2016) did not find differences in PTH concentrations among the BMI groups and PTH did not correlate with BMI in either sex. They suggested that the relationship between

25(OH)D and PTH may be altered in obesity. There are also studies where body weight was a strong predictor of PTH, but 25(OH)D had little or no relation with PTH. Obese young Finnish adults had lower total and measured free 25(OH)D and slightly higher PTH than their normal-weight peers, and free 25(OH)D was associated with obesity-related parameters (Holmlund-Suila *et al.* 2016).

Studies on the association of total or free 25(OH)D with bone have revealed contradictory results. Study III is the first study to examine the direct association of free and bioavailable 25(OH)D with several bone traits measured with pQCT. Obese men and women had higher trabecular density and CSI but slightly lower cortical density. We found that total 25(OH)D was a significant but weak negative determinant of trabecular density of the distal radius, and free and bioavailable 25(OH)D were negative determinants of tibial shaft CSI. Free and bioavailable 24(OH)D were negatively associated with cortical density of the radial shaft. In normal-weight women or men, no significant association was found between vitamin D metabolites and bone traits. Furthermore obesity was associated with lower concentrations of bone turnover markers in both men and women.

In a study by Johnsen *et al*. (2014), free and bioavailable but not total 25(OH)D, correlated with BMD, and when an adjustment with *GC* genotype-specific constants was made the association improved. Jemielita *et al*. (2016) found racial differences in the association of 25(OH)D with bone. Total and calculated free 25(OH)D were positively associated with bone traits measured with pQCT in black subjects but not in white subjects. However, to determine DBP concentration they used a monoclonal antibody-based method, which has been shown to give false results, especially among dark-skinned populations (Bouillon *et al.* 2014).

Walsh *et al*. (2016) found that whole-body, hip, and spine BMD as well as distal radius and tibia trabecular density were higher in the obese and overweight groups than in the normal-weight group. Bone turnover was also higher in obese subjects, contrary to the results of Study III. They suggested that lower 25(OH)D in obesity could be a sign of vitamin D deficiency, but the positive skeletal effects of obesity, such as increased oestrogen synthesis, could reverse the effect on bone. However, women of Study III were premenopausal and with sufficient oestrogen production from the ovaries. A potential explanation for the negative associations of free and bioavailable 25(OH)D concentrations with some bone traits in obese women could be altered vitamin D metabolism due to higher DBP and lower free and bioavailable 25(OH)D. Obese women were more frequent smokers and they exercised less than normal-weight women. Higher

DBP concentrations in the obese could also mean greater GC-MAF production and moreover osteoclast activation.

Obesity is associated with stronger bones and prevents osteoporosis and fractures, particularly hip fractures (Johansson *et al.* 2014). One reason is greater body weight, which creates additional loading on the weight-bearing skeleton. Also the large amount of adipose tissue may produce more oestrogen which, in turn, has a major impact especially on female skeleton (Järvinen *et al.* 2003). Excess weight in adolescence is known to be associated with larger bone cross-section and to modulate also BMD in adulthood (Uusi-Rasi *et al.* 2012). However, the amount and intensity of physical activity are the major determinants of bone loading (Weeks *et al*. 2008).

6.6 Evaluation of the studies

6.6.1. Study design, population, and sample size

The cross-sectional design of the studies allows only observation of the situation at a specific time point and prevents determination of causality.

Because the data from Studies II and III originate from a study focusing on phosphorus and bone, the power calculations were not based on vitamin D outcomes. When examining bone traits, the genders must be analysed separately. In Studies I-III, the proportion of boys/men relative to girls/women was much smaller. In Study I, almost 70 % of the subjects were girls, and in Studies II and III the proportion of women was approximately 65%.

The study population of children and adolescents in Study I was rather challenging. Firstly, the subjects were derived from two cohorts (Optiford study and School-based study) collected over a wide time frame (2003-2008). Secondly, the age of the subjects varied from 8 to 19 years. Especially when the data were divided into GC genotype groups, puberty status in both girls and boys was not even in the groups. Genotype

distribution was also uneven; the number of subjects was low, particularly in the rare genotype *GC* 2/2 and in boys.

6.6.2 Methods

At the time when Studies I and II were conducted, there were no Finnish genotype database and the CEU database (Northern Europeans from Utah) was used instead. Since then, a database that covers the gene variation in the Finnish population has been established. Finland's gene pool has been shaped by several bottlenecks during population history. SISu (http://www.sisuproject.fi/) was established in 2014 to build a specific reference dataset of sequence variants in Finns. The current version covers SNPs and indels from exomes of over 10 000 individuals sequenced in disease-specific and population genetic studies. The Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org/) is an association of investigators seeking to aggregate and harmonize exome sequencing data from a wide variety of large-scale sequencing projects, and to make summary data available to the wider scientific community. The genotyping was conducted with Real Time PCR using probes from Allogene (Study I) and by using TaqMan chemistry on an automatic sequence-detection instrument (Study II). In Study I, the common *GC* SNPs rs4588 and rs7041 were selected. These SNPs are known to have variation and to be associated with 25(OH)D measures in other populations. In Study II, SNPs covering the DBP gene region were selected from the International HapMap Project database. The CEU population was used as a reference when selecting the SNPs, again because no Finnish database existed at the time of the study.

There are several methods for measuring 25(OH)D in serum, as described in the review of the literature and the methods have developed considerably over the years. In this thesis, the measurements were conducted with IDS ELISA (Studies II and III) and HPLC (Study I). The Vitamin D External Quality Assessment Scheme (DEQAS) has revealed considerable differences among different methods (both within and between laboratories), raising concerns about the comparability and accuracy of different assays and laboratories.

At the time that the samples for Studies II and III were analysed (2010), the analysing laboratory was in the process of completing the DEQAS certificate to guarantee reproducibility of analyses. The certificate was received in 2012.

LC-MS/MS analyses have shown that immunoassays display interference of $24,25(OH)_2D$, especially at higher 25(OH)D concentrations, yielding overly high 25(OH)D values (Malmström *et al.* 2017), whereas no interference with 25(OH)D measurements occurs when using HPLC or LC-MS/MS, LC-MS/MS and HPLC can measure 25-hydroxycholecalciferol ($25(OH)D_3$) and 25-hydroxyergocalciferol ($25(OH)D_2$) separately. They also allow detection of other vitamin D metabolites such as 24,25-dihydroxyvitamin D ($24,25(OH)_2D$). Therefore, these two methods are considered the gold standard for measurement of vitamin D metabolites.

Several studies have found that calculating free 25(OH)D overestimates the concentrations relative to directly measuring free 25(OH)D (Schwartz 2014, Aloia *et al.* 2015, Lee *et al.* 2015, Alzaman 2016, Sollid 2016). The overestimation seems to be higher in blacks and especially when using a monoclonal antibody–based assay for measuring DBP (Bouillon *et al.* 2014, Aloia *et al.* 2015, Alzaman 2016). The monoclonal antibody-based assay appears not to recognize the 1F allele in DBP, which is common in African Americans, and therefore, several studies using monoclonal antibodies in their DBP analyses are not reliable. The direct method for measuring 25(OH)D is, in any case, novel and unpredictable. There are some limitations when calculating free and bioavailable 25(OH)D concentrations. First, the affinity constants of DBP that Arnauld & Constans (1993) used in the calculation of free and bioavailable 25(OH) concentrations are derived from one individual's human serum sample. It is not known whether there are post-translational modifications of DBP that could affect its affinity for vitamin D metabolites. Second, the GC affinity constant for 25(OH)D in the literature varies widely (Bikle *et al.* 1986). Furthermore, because the full sequence of the DBP is not known, there might also be isoforms other than the common ones. According to Arnauld's analysis, binding affinity of these rarer isoforms may vary as much as 12-fold (Arnauld & Constans 1993, Malmström *et al.* 2017). A certified method for measuring total 25(OH)D and method using polyclonal antibodies in DBP measurement makes the calculation of free 25(OH)D values in Studies II and III rather reliable.

DXA is the most common method to assess BMD and BMC, and it is considered the gold standard in all age groups. However, it has several limitations. DXA measures areal BMD, thus two dimensions, and not true volumetric density like pQCT. aBMD (g/cm^2)

consists of bone mass or BMC per bone area of a three-dimensional structure. Skeletal growth in children leads to a much higher increase in volume than in bone area and therefore, young children have proportionally larger area in relation to bone volume compared with older children (Pezzuti *et al.* 2017). Minimal exposure to radiation especially in children is also an advantage of pQCT-measurements. The use of pQCT in bone measurements provides relevant data for trabecular and cortical BMD as well as for bone geometry, size, and mass (Sievänen *et al.* 1998). In this thesis, several bone traits were measured with pQCT in non-weight-bearing radius and weight-bearing tibia in different age subjects (children and adolescents as well as adults. In Study I, the skeletal age of the subjects was not determined at the time when the studies were conducted, and therefore the only classification method was the puberty stage and age.

Extensive background data, including sunshine exposure, dietary intakes of vitamin D and calcium, vitamin D intake from supplements, and collecting data on of physical activity and measurement of several established biomarkers are strengths of this thesis. In addition, there was a large population-based sample of both women and men in Studies II and III. Recording the influence of bone-loading activity on bone traits and history of physical activity in children and adolescents as well as in the adults, could have provided important additional information for the bone analysis. Because weight and height are strong determinants of bone size related traits (Uusi-Rasi 2010) and the purpose was to study the other factors that may affect bone, only the bone parameters that were independent of body size were included in the Study III. The measurement of fat percentage, waist circumference or body composition would have been a better alternative for measuring obesity than BMI in Study III. Menopause was determined in Studies II and III.

7 SUMMARY AND CONCLUSIONS

At the time that Study I was conducted, it was the first study to examine the association between *GC* genotype and bone health in children and adolescents. Based on the results from Studies I and II, it can be proposed that 25(OH)D and PTH concentrations differ among *GC* genotypes and that variation exists in BMD and bone strength among genotypes in children and adolescents. Variation in 25(OH)D among genotypes suggests differences in vitamin D utilization. In Study I, more associations between GC genotype and bone traits were found in boys, although an association of GC with SSI was found in girls. This may be due to gender differences in the phase of skeletal development and also the low number of boys in the study. Based on the outcomes of Study I, it can be concluded that DBP may be one factor taking part in bone mass accrual during adolescence. However, the cross-sectional design prevented from establishing a causal relationship between *GC* genotype, 25(OH)D, PTH, and bone accrual. In the future, DBP polymorphism could be useful in detecting individuals who are more vulnerable to vitamin D deficiency, especially while their skeleton is still developing.

The findings from Study II indicate that genetic polymorphisms of DBP and free metabolites of 25(OH)D may be relevant when vitamin D status and vitamin D metabolism are evaluated. These findings suggest that DBP genotype may influence vitamin D metabolism through multiple pathways. The mechanism may be through differences in plasma 25(OH)D directly suppressing PTH production and secretion in the parathyroid gland or through differences in free 25(OH)D, which may modulate the availability of 1,25(OH)D, suppressing PTH. Further investigation of the effect of DBP on free 25(OH)D is warranted.

The observed associations between obesity and total, free, and bioavailable 25(OH)D, DBP, and PTH in Study III and similar results from previous studies suggest that obese individuals differ from their normal-weight peers in vitamin D metabolism. Moreover, there was negative association of free and bioavailable vitamin D metabolites with some pQCT-measured bone traits in obese women. In the future, obesity and genetic determinants of vitamin D should be taken into account to detect individuals susceptible to vitamin D deficiency who could benefit from vitamin D supplementation.

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Elisa Saarnio

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